UNITED STATES ENVIRONMENTAL PROTECTION AGENCY
WASHINGTON, D.C. 20460

OFFICE OF CHEMICAL SAFETY AND POLLUTION PREVENTION

**MEMORANDUM**

**DATE:** September 16, 2020

**SUBJECT:** Dicamba Mutagenicity Data Evaluation Records (DERs)

**PC Code:** 029801
**Decision Nos.:** 562443
**Petition No.:** N/A
**Risk Assessment Type:** N/A
**TXR No:** 0058082
**MRID Nos.:** 51129101, 51129102, 51129103, 51129104, 51129105, 51129106, 51129107, 51129108, 51129109

**DP Barcodes:** D458715
**Registration No.:** 7969-132
**Regulatory Action:** N/A
**Case No.:** N/A
**CAS No.:** 1918-00-9
**40 CFR:** N/A

**FROM:** Sarah Dobreniecki Ph.D., Biologist
Risk Assessment Branch VII
Health Effects Division, 7509P
Sarah Dobreniecki

**THROUGH:** Michael Metzger, Branch Chief
Risk Assessment Branch V/VII
Health Effects Division, 7509P
Michael R. Metzger

**TO:** Margaret Hathaway, Risk Manager Reviewer
Reuben Baris, Risk Manager (PY1 S-7227)
Registration Division (7505P)

## I. CONCLUSIONS

The Registration Division (RD) asked the Health Effects Division (HED) to review the data submission regarding dicamba and mutagenicity. The conclusions of this submission are summarized below.

## II. RESULTS/DISCUSSION

1) Barfield, W. (2019) Dicamba – Crl:CD(SD) rat *in vivo* comet test. Envigo CRS Ltd., Alconbury, Huntingdon, Cambridgeshire, UK. Laboratory Study No.: XB29VC, February 14, 2019. MRID 51129101. Unpublished.

**EXECUTIVE SUMMARY:** In a non-guideline, *in vivo* comet test (MRID 51129101), groups of six Crl:CD(SD) male rats/dose group were administered dicamba (89.8% a.i., batch # P.MG2726410) in aqueous 0.5% methylcellulose via oral gavage (dose volume 10 mL/kg) at dose levels of 0, 37.5, 75, or 150 mg/kg/day; two doses were administered approximately 24 hours apart. Approximately two hours after the second dose, the rats were euthanized, and sections of the liver and duodenum were excised and prepared for comet analysis. Additionally, one group of three rats was administered dicamba as above at 150 mg/kg/day for plasma analysis; blood samples were collected one and two hours after the second dose. Another group of three rats was administered ethyl methanesulfonate (EMS; positive control) in water via oral gavage (dose volume 10 mL/kg) at 200 mg/kg and euthanized after approximately three hours; sections of liver and duodenum were excised and prepared for comet analysis.

A preliminary test was performed to determine the maximum tolerated dose (MTD) prior to the comet test. First, a group of two Crl:CD(SD) male rats was administered dicamba as above at a dose level of 500 mg/kg/day. These rats exhibited clinical signs of severe toxicity and were euthanized on Day 1. Because the MTD was exceeded, a second group of two rats was administered dicamba as above at a dose level of 150 mg/kg/day; the MTD was established at this dose level.

The plasma analysis confirmed dicamba was systemically available following two administrations by oral gavage. The preliminary test established an MTD at 150 mg/kg/day. DNA analysis by the comet test resulted in no effects of treatment on the liver at up to 150 mg/kg/day; the positive control (EMS) induced an expected increase in tail intensity with no increase in hedgehog cells. In the duodenum, the marked increase in tail intensity at 37.5 and 75 mg/kg/day would indicate genotoxicity. However, the marked increase in hedgehog cells observed at these doses, indicating cytotoxicity, were not corroborated by microscopic findings. Therefore, the Reviewers consider the results of this study equivocal.

This study is classified as **acceptable / non-guideline**.

**COMPLIANCE:** Signed and dated Data Confidentiality, GLP Compliance, Flagging, and Quality Assurance statements were provided.

2) Herring, T. (2019) Dicamba – Crl:CD(SD) rat histopathological follow-up study. Envigo CRS Ltd., Alconbury, Huntingdon, Cambridgeshire, UK. Laboratory Study No.: NS52VW, February 15, 2019. MRID 51129102. Unpublished.

**EXECUTIVE SUMMARY:** In a concurrently-reviewed, non-guideline, *in vivo* comet test (MRID 51129101), increased DNA strand breaks accompanied by increased numbers of hedgehog cells were observed in the duodenum of Crl:CD(SD) male rats administered dicamba in aqueous 0.5% methylcellulose via oral gavage (dose volume 10 mL/kg) at dose levels of 37.5 or 75 mg/kg/day. The present study was performed in order to further investigate the effects on point-of-contact tissues. In this non-guideline, histopathological follow-up study (MRID 51129102), groups of five Crl:CD(SD) male rats were administered dicamba (89.8% a.i., batch # P.MG2726410) in aqueous 0.5% methylcellulose via oral gavage (dose volume 10 mL/kg) at dose levels of 0, 37.5, or 75 mg/kg/day; two doses were administered approximately 24 hours apart. At approximately 2, 6, 24, and 48 hours after the second dose, the rats were euthanized, and sections of the stomach and duodenum were excised, fixed, and routinely processed for microscopic pathological examinations. Sections were visualized with hematoxylin and eosin or by using the terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) and active caspase 3 stains to detect cytotoxicity, necrosis, and/or apoptosis.

There was no treatment-related cytotoxicity, necrosis, or apoptosis at the dose levels administered in this study.

This study is classified as **acceptable / non-guideline**.

**COMPLIANCE:** Signed and dated Data Confidentiality, GLP Compliance, Flagging, and Quality Assurance statements were provided.

3) Barfield, W. (2020) Dicamba techn. (BAS 183 H; SAN837 techn.): Follow up study to determine potential *ex vivo* effects during comet tissue processing. Covance CRS Ltd., Alconbury, Huntingdon, Cambridgeshire, UK. Laboratory Study No.: MM44NB, March 30, 2020. MRID 51129103. Unpublished.

**EXECUTIVE SUMMARY:** In a concurrently-reviewed, non-guideline, *in vivo* Comet test (MRID 51129101), increased DNA strand breaks accompanied by increased numbers of hedgehog cells were observed in the duodenum of Crl:CD(SD) male rats administered dicamba in aqueous 0.5% methylcellulose via oral gavage (dose volume 10 mL/kg) at dose levels of 37.5 or 75 mg/kg/day. In order to further investigate potential *ex vivo* effects on point-of-contact tissues, the present study was performed. In this non-guideline, mechanistic follow-up study (MRID 51129103), groups of three Crl:CD(SD) male rats were administered dicamba (89.8% a.i., batch # P.MG2726410) in aqueous 0.5% methylcellulose via oral gavage (dose volume 10 mL/kg) at dose levels of 0 or 75 mg/kg/day; two doses were administered approximately 24 hours apart. At approximately 0.5, 1, 2, 4, or 6 hours after the second dose, the rats were euthanized; control rats were euthanized 2 hours after the second administration of vehicle. A seventh group of three rats were administered three doses at 25 mg/kg at 30-minute intervals; this dosing was performed twice 24 hours apart, and the rats were euthanized 2 hours after the second dosing was completed. A positive control group was administered ethyl methanesulfonate in purified water at 200 mg/kg and euthanized 3 hours after the single dose. Single cell suspensions of the duodenum and liver were prepared for comet analysis.

Comet test data showed increased % tail intensity accompanied by increased hedgehog cells across some, but not all, time points. The positive control generated data consistent with expected findings. Intracellular pH data was subjective, not quantifiable, and inconclusive.

Mincing solution pH and osmolality was not affected by incubation times or cell suspension preparation. There were no microscopic findings of cytotoxicity, necrosis, or apoptosis.

This study is classified as **acceptable / non-guideline**.

**COMPLIANCE:** Signed and dated Data Confidentiality, GLP Compliance, Flagging, and Quality Assurance statements were provided.

4) Hilton, A. (2020) [$^{14}$C]-Dicamba: duodenum kinetics in rats. Covance CRS Ltd., Alconbury, Huntingdon, Cambridgeshire, UK. Laboratory Study No.: MT42NJ, February 28, 2020. MRID 51129104. Unpublished.

**EXECUTIVE SUMMARY:** In a concurrently-reviewed, non-guideline, *in vivo* comet test (MRID 51129101), increased DNA strand breaks accompanied by increased numbers of hedgehog cells were observed in the duodenum of Crl:CD(SD) male rats administered dicamba in aqueous 0.5% methylcellulose via oral gavage (dose volume 10 mL/kg) at dose levels of 37.5 or 75 mg/kg/day. The present study was performed to obtain the absorption kinetics and rates and routes of excretion of dicamba following oral administration to rats. In this non-guideline, absorption kinetics follow-up study (MRID 51129104), groups of four Crl:CD(SD) male rats were administered [$^{14}$C]-dicamba (radiochemical purity 99%, batch # WJE-I-57) in aqueous 0.5% methylcellulose via oral gavage (dose volume 10 mL/kg) at a dose level of 75 mg/kg/day; two doses were administered approximately 24 hours apart. At approximately 0.5, 1, 2, 4, or 6 hours after the second dose, the rats were euthanized. Whole blood and plasma, duodenum sections, mincing solutions and scrapings, liver sections, and urine samples were obtained at each time point and analyzed for radioactivity.

No clinical signs of toxicity were reported.

[$^{14}$C]-Dicamba is rapidly absorbed after oral gavage administration. The maximum concentration of [$^{14}$C]-dicamba in whole blood was observed at 0.5 hours after the second dose and declined steadily to the final 6-hour sample. Similarly, the maximum concentration of [$^{14}$C]-dicamba in plasma was observed at 0.5 hours after the second dose and declined steadily to the final 6-hour sample. Plasma concentrations were consistently greater than those in whole blood.

In the duodenum sections, mean total radioactivity and mean concentration of radioactivity were greatest at 0.5 hours after the second dose and declined with time. Concentrations were still measurable at 6 hours after the second dose. Concentrations in duodenum sections were greatest in section A (immediately after the stomach) and declined from sections A to B and generally declined from sections B to C between 0.5-2 hours after the second dose. At 4 and 6 hours, mean concentrations were generally similar across all sections. Mean total radioactivity and mean concentrations of radioactivity in the mincing solutions and scrapings were less than those found in the duodenum sections and followed the same time course, approaching the limit of quantitation at 6 hours. Mean concentrations of radioactivity in the liver sections were greatest at 0.5 hours after the second dose and declined with time to 6 hours.

Radioactivity was detectable in the urine at 1 hour following the first dose with the greatest concentration at 4 hours. Radioactivity concentrations fell to the lowest concentration at 24 hours after the first dose. Following the second dose, radioactivity began to rise, with the greatest concentration at 2 hours after the second dose and falling with time to the final collection at 6 hours.

This study is classified as **acceptable / non-guideline**.

**COMPLIANCE:** Signed and dated Data Confidentiality, GLP Compliance, Flagging, and Quality Assurance statements were provided.

5) Ueda, M. (2020) Dicamba techn. (BAS 183 H; SAN837 techn.): transgenic mice (Muta™Mouse) gene mutation assay. BioSafety Research Center, Inc., Shizuoka, Japan. Laboratory Study ID: 886458, March 13, 2020. MRID 51129105. Unpublished.

   Ueda, M. (2020) Dicamba techn. (BAS 183 H; SAN837 techn.): dose range-finding study for transgenic mice (Muta™Mouse) gene mutation assay. BioSafety Research Center, Inc., Shizuoka, Japan. Laboratory Study ID: 886460, March 10, 2020. MRID 51129106. Unpublished.

   Blum, M. (2020) Validation of an analytical method for the analysis of BAS 183 H (dicamba techn.) in powdered diet, CRF-1 (Oriental Yeast) using HPLC-UV (Control procedure 97/0267_03). BASF SE, Experimental Toxicology and Ecology, Ludwigshafen, Germany. Laboratory Study ID: 887672, February 3, 2020. MRID 51129107. Unpublished.

   Bangert, L. (2020) BAS 183 H (Dicamba techn.): homogeneity and concentration control analysis in powdered diet CRF-1 (Oriental Yeast). BASF SE, Experimental Toxicology and Ecology, Ludwigshafen, Germany. Laboratory Study ID: 887680, January 30, 2020. MRID 51129108. Unpublished.

   Wagner, I. (2020) BAS 183 H (Dicamba techn.): stability analysis in powdered diet CRF-1 (Oriental Yeast). BASF SE, Experimental Toxicology and Ecology, Ludwigshafen, Germany. Laboratory Study ID: 887679, February 11, 2020. MRID 51129109. Unpublished.

**EXECUTIVE SUMMARY:** In a non-guideline, gene mutation assay (MRID 51129105), groups of seven male Muta™Mouse ($CD_2$-LacZ80/HazfBR) mice/dose level were administered dicamba (89.8% a.i., batch # P.MG2726410) in the diet at dose levels of 0, 1200, 3000, or 7000 ppm (equivalent to 0, 176.4, 431.1, and 924.9 mg/kg/day) for 28 consecutive days. After a three-day period for mutations to become fixed in the genomic DNA, the mice were euthanized on Day 31. A positive control group of seven male mice were administered *N*-ethyl-*N*-nitrosourea (ENU) in 1/15 mol/L sodium phosphate buffer (pH 6.0) by i.p. injection (dose volume 10 mL/kg) at a dose level of 100 mg/kg/day; two doses were given approximately

24 hours apart on Days 3 and 4. After ten days, these mice were euthanized on Day 14. The duodenum was examined for genomic DNA mutations induced by test substance exposure.

There were no effects of treatment on clinical signs of toxicity, absolute or relative (to body) duodenum weights, or necropsy or microscopic findings. There were no changes in absolute or relative (to body) duodenum weights in the ENU-treated group.

In the 7000 ppm group, there were decreases in body weight on Days 15 (↓6%) and body weight gain during Days 1-31 (−0.1 g treated vs 0.7 g control). Additionally, at 7000 ppm, food consumption was decreased by 22% during Days 1-3. In the positive control group, there was a decrease in body weight during Days 1-14 (↓8%).

Administration of dicamba in the diet did not increase mutant frequency. All mean values fell within the 95% confidence range ($14.4 \times 10^{-6}$ to $86.9 \times 10^{-6}$) calculated from the historical control data. The positive control (ENU) produced a marked 10.6-fold increase in the mutant frequency.

This study is classified as **acceptable / non-guideline**.

**COMPLIANCE:** Signed and dated Data Confidentiality, GLP Compliance, Flagging, and Quality Assurance statements were provided.

# DATA EVALUATION RECORD

DICAMBA

Study Type: OCSPP Non-Guideline; *In Vivo* Comet Test
(Single Cell Gel Electrophoresis Assay) in Rats

EPA Contract No. EP-W-16-018
Task Assignment No. 34-3-001 (MRID 51129101)

Prepared for
Health Effects Division
Office of Pesticides Program
U.S. Environmental Protection Agency
2777 South Crystal Drive
Arlington, VA 22202

Prepared by
CDM | CSS-Dynamac
Joint Venture
10560 Arrowhead Dr., Suite 500
Fairfax, VA 22030

| | | |
|---|---|---|
| Primary Reviewer: | Signature: | |
| Michael E. Viana, Ph.D. | Date: | 08/05/2020 |
| Secondary Reviewer: | Signature: | |
| Sarah E. Saucier, Ph.D. | Date: | 08/20/2020 |
| Quality Assurance: | Signature: | |
| Scott D. Studenberg, Ph.D., DABT | Date: | 08/25/2020 |
| Project Manager: | Signature: | |
| Michael E. Viana, Ph.D. | Date: | 08/31/2020 |

This Data Evaluation Record may have been altered by the Health Effects Division subsequent to signing by CDM/CSS-Dynamac Joint Venture personnel. Contractor's role did not include establishing Agency policy.

**EPA Reviewer:** Sarah Dobreniecki **Signature:** Sarah Dobreniecki
**Risk Assessment Branch VII, HED (7509P)** **Date:** 9/16/2020
**Template version 02/06**

# DATA EVALUATION RECORD

**STUDY TYPE:** *In Vivo* Comet Test (Single Cell Gel Electrophoresis Assay) in Rats; OCSPP Non-Guideline; OECD 489.

**PC CODE:** 029801

**DP BARCODE:** D458715

**TXR #:** 0058082

**TEST MATERIAL (PURITY):** Dicamba (89.8% a.i.)

**SYNONYMS:** SAN837; 3,6-dichloro-2-methoxybenzoic acid

**CITATION:** Barfield, W. (2019) Dicamba – Crl:CD(SD) rat *in vivo* comet test. Envigo CRS Ltd., Alconbury, Huntingdon, Cambridgeshire, UK. Laboratory Study No.: XB29VC, February 14, 2019. MRID 51129101. Unpublished.

**SPONSORS:** Syngenta, Ltd., Jealott's Hill International Research Centre, Bracknell, Berkshire, UK

BASF Corp., 100 Park Avenue, Florham Park, NJ

Rotam Agrochem International Co., Ltd., 26/F, E-Trade Plaza, 24 Cheung Lee Street, Chai Wan, Hong Kong

**EXECUTIVE SUMMARY:** In a non-guideline, *in vivo* comet test (MRID 51129101), groups of six Crl:CD(SD) male rats/dose group were administered dicamba (89.8% a.i., batch # P.MG2726410) in aqueous 0.5% methylcellulose via oral gavage (dose volume 10 mL/kg) at dose levels of 0, 37.5, 75, or 150 mg/kg/day; two doses were administered approximately 24 hours apart. Approximately two hours after the second dose, the rats were euthanized, and sections of the liver and duodenum were excised and prepared for comet analysis. Additionally, one group of three rats was administered dicamba as above at 150 mg/kg/day for plasma analysis; blood samples were collected one and two hours after the second dose. Another group of three rats was administered ethyl methanesulfonate (EMS; positive control) in water via oral gavage (dose volume 10 mL/kg) at 200 mg/kg and euthanized after approximately three hours; sections of liver and duodenum were excised and prepared for comet analysis.

A preliminary test was performed to determine the maximum tolerated dose (MTD) prior to the comet test. First, a group of two Crl:CD(SD) male rats was administered dicamba as above at a dose level of 500 mg/kg/day. These rats exhibited clinical signs of severe toxicity and were euthanized on Day 1. Because the MTD was exceeded, a second group of two rats was

administered dicamba as above at a dose level of 150 mg/kg/day; the MTD was established at this dose level.

The plasma analysis confirmed dicamba was systemically available following two administrations by oral gavage. The preliminary test established an MTD at 150 mg/kg/day. DNA analysis by the comet test resulted in no effects of treatment on the liver at up to 150 mg/kg/day; the positive control (EMS) induced an expected increase in tail intensity with no increase in hedgehog cells. In the duodenum, the marked increase in tail intensity at 37.5 and 75 mg/kg/day would indicate genotoxicity. However, the marked increase in hedgehog cells observed at these doses, indicating cytotoxicity, were not corroborated by microscopic findings. Therefore, the Reviewers consider the results of this study equivocal.

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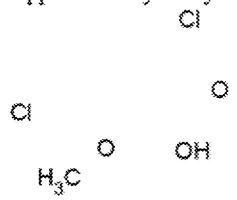
# I. MATERIALS AND METHODS

## A. MATERIALS

**1. Test material:** Dicamba

| | |
|---|---|
| **Description:** | White solid |
| **Batch #:** | P.MG2726410 |
| **Purity:** | 89.8% a.i. |
| **CAS # of TGAI:** | 1918-00-9 |
| **Stability:** | Approximately two years stored at <30°C |
| **Structure:** | |




**2. Positive control:** Ethyl methanesulfonate (EMS; lot/batch # and source not reported)

**3. Vehicle:** Aqueous 0.5% (w/v) methylcellulose (batch #: MKBN2740V; Sigma).

**4. Test animals**

| | |
|---|---|
| **Species:** | Rat (male only) |
| **Strain:** | Crl:CD(SD) |
| **Age / weight at Day 1:** | Preliminary test: approximately 7-9 weeks / 200-206 g<br>Comet test: approximately 7-8 weeks / 181-205 g |
| **Source:** | Charles River UK Ltd. (Margate, Kent, England) |
| **Housing:** | It was stated the groups were kept in cages; however, additional information (type of cage and rats per cage) were not provided. Rats were provided untreated wood chew blocks and a red plastic shelter for environmental enrichment. |
| **Diet:** | Pelleted Envigo Teklad 2014C diet, *ad libitum* |
| **Water:** | Tap water, *ad libitum* |
| **Environmental conditions** | |
| **Temperature:** | 20-24°C |
| **Humidity:** | 40-70% |
| **Air changes:** | Not provided |
| **Photoperiod:** | 12 hours light/12 hours dark |
| **Acclimation period:** | 5 days minimum |

## B. STUDY DESIGN

**1. In-life dates:** Not reported

**2. Animal assignment:** Following receipt, the rats were weighed and randomly assigned to the groups in Table 1. No additional information was reported.

| **TABLE 1:** Study design [a] | | | |
|---|---|---|---|
| **Group** | **Dose (mg/kg/day)** | **Concentration (mg/mL)** | **# males** |
| **Preliminary test** | | | |
| 1 | 500 | 50 | 2 |
| 2 | 150 | 15 | 2 |
| **Comet test** | | | |
| 1 | 0 | 0 | 6 |
| 2 | 37.5 | 3.75 | 6 |
| 3 | 75 | 7.5 | 6 |
| 4 | 150 | 15 | 6 |
| 4 [b] | 150 | 15 | 3 |
| 5 [c] | 200 | 20 | 3 |

a Data were obtained from page 22 of MRID 51129101.
b Dicamba satellite group for plasma analysis.
c EMS in purified water.

3. **Dose-selection rationale:** The doses were selected on the basis of the preliminary test. No further information was reported.

4. **Preparation of test formulations:** For each dose formulation, an appropriate amount of the test substance (adjusted for purity) was weighed and ground in a mortar with a pestle; vehicle (aqueous 0.5% w/v methylcellulose) was added and mixed to form a paste. Additional vehicle was added to produce a smooth, pourable suspension, and the suspension was brought up to final volume with vehicle and homogenized.

   EMS (positive control) was dissolved in purified water to form a 20 mg/mL solution.

   Homogeneity and stability analyses were performed on 1 and 200 mg/mL formulations with ambient (15-25°C) and refrigerated (2-8°C) storage. The formulations were stored in two bottles each. One bottle of each concentration was kept at ambient temperature and stirred for 20 minutes to represent a zero-hour sample time; the formulation was stirred continuously and single samples from the top, middle, and bottom were removed at 0, 1, and 2 hours. The remainder of the bottle was stored for one day at ambient temperature, remixed, and sampled again as above. The second bottle of each concentration was stored refrigerated on receipt, warmed to ambient temperature after one or eight days of refrigerated storage, mixed by 20 inversions followed by at least 20 minutes of stirring, and sampled as above. Concentration analyses were performed on freshly prepared suspensions; single samples from the top, middle, and bottom (vehicle was sampled twice from the middle) were analyzed.

   **Results**

   **Homogeneity analyses (%CV):** 0.17-4.18%

**Stability analysis (% of time 0):** 100.7-107.1% following one day storage at ambient temperature; 101.7-106.6% following eight days storage refrigerated.

**Concentration analysis (% of nominal):** 100.1-102.2%

The analytical data indicated that the mixing procedure was adequate and that the variance between nominal and actual dosage to the animals was acceptable.

5. **Dose administration:** The dose suspensions and positive control were administered by oral gavage at a dose volume of 10 mL/kg; the volumes were calculated from the most recent body weights. The dose suspensions were administered twice approximately 24 hours apart, and the positive control was administered once.

6. **Statistics:** Data for the median tail intensity values from the comet tests for the test substance suspensions and the positive control were analyzed separately. For the test substance suspensions, Bartlett's test for homogeneity of variances was applied; if Bartlett's test was not significant ($p>0.01$), parametric analyses were performed. First, the F1 approximate test was performed; if the F1 test was not significant ($p>0.01$), Williams' test for a monotonic trend was performed. If the F1 test was significant ($p\leq0.01$), Dunnett's test was used to compare the treated groups to the control. If Bartlett's test was significant ($p\leq0.01$), logarithmic and square-root transformations were applied (0.001 was added to any zero values prior to logarithmic transformation) and Bartlett's test was performed again. If Bartlett's test was still significant, non-parametric analyses were performed. First, the H1 approximate test was performed; if the H1 test was not significant ($p>0.01$), Shirley's test for a monotonic trend was performed. If the H1 test was significant ($p\leq0.01$), Steel's test was performed.

   For the positive control, Bartlett's test was applied; if Bartlett's test was not significant, the positive control was compared to the control with a t-test (parametric analyses). If Bartlett's test was significant, logarithmic and square-root transformations were performed as above. If Bartlett's test was still significant, the positive control was compared to the control with the Wilcoxon rank sum test.

   Significance was denoted at $p\leq0.05$.

   The Reviewers consider the statistical analyses used appropriate.

7. **Comet test:** The Single Cell Gel Electrophoresis (SCGE) assay (Comet test) is a rapid, visual, and quantitative technique for measuring DNA strand breakage in individual mammalian cells. Cell suspensions isolated from *in vivo*-treated animal tissues are embedded in agarose gel on microscope slides. The cells are lysed with detergent and high salt solution to rupture the cell membranes, extract the nuclear proteins, and leave the supercoiled DNA in a nucleus-type structure (nucleoid). Exposure to a strongly alkaline buffer causes the supercoiled DNA to relax and unwind. Because DNA carries a net negative charge, the relaxed loops and single strand fragments migrate toward the anode during electrophoresis. If DNA strand breaks occur during the chemical insult, the open

loops or fragments migrate further within the gel. After electrophoresis, the DNA is neutralized and stained with a nucleic acid-specific dye. The cells are visualized and quantitated by using fluorescence microscopy linked to an image analysis system.

8. **Evaluation and interpretation of results:** The following statements were provided regarding evaluation and interpretation of the comet test results.

The following criteria were applied for assessment of assay acceptability:

- The concurrent vehicle control data were considered acceptable for addition to the historical control database;
- Concurrent positive control data indicated responses that corresponded to the historical positive control data and were significantly increased compared to the concurrent vehicle control;
- An MTD was achieved; and
- An adequate number of cells and dose levels were analyzed.

Providing that all acceptability criteria are fulfilled, a test substance is considered clearly positive if: a) at least one of the test doses exhibits a statistically significant increase compared with the concurrent vehicle control, b) the increase is dose-related if evaluated with an appropriate trend test, and c) any of the results are outside the distribution of the historical vehicle control data. If all of these criteria are met, the test substance is considered able to induce DNA strand breakage in the tissues studied in this test system.

Providing that all acceptability criteria are fulfilled, a test substance is considered clearly negative if: a) none of the test concentrations exhibits a statistically significant increase compared with the concurrent vehicle control, b) there is no concentration-related increase if evaluated with an appropriate trend test, and c) all results are inside the distribution of the historical vehicle control data. If all of these criteria are met, the test substance is considered unable to induce DNA strand breakage in the tissues studied in this test system.

There is no requirement for verification of a clearly positive or negative response.

In case the response is neither clearly negative nor clearly positive as described above and in order to assist in establishing the biological relevance of a result, the data should be evaluated by expert judgement and/or further investigations. Scoring additional cells (as appropriate) or performing a repeat experiment possibly with modified experimental conditions (*e.g.*, dose spacing, other routes of administration, other sampling times, or other tissues) could be useful.

In rare cases, even after further investigations, the data set will preclude making a conclusion of positive or negative results and will, therefore, be concluded as equivocal. To assess the biological relevance of a positive or equivocal result, cytotoxicity at the target tissue should also be discussed. Histopathological information can help in the interpretation of a positive result in the comet assay.

Furthermore, cell death is associated with increased levels of DNA strand breaks. In the comet assay, the microscopical image resulting from excessively damaged cells, *e.g.*, necrotic or apoptotic cells, are comets with small or non-existent heads and large diffuse tails, sometimes referred to as 'hedgehog' or 'ghost' comets.

Careful interpretation of increased or decreased % tail DNA in the presence of severe cytotoxicity is therefore essential (OECD 489).

## C. METHODS

1. **Observation:** The rats were observed twice daily for mortality and morbidity. Rats found *in extremis* were humanely euthanized.

2. **Body weight:** All rats were weighed following arrival and on each day of dosing.

3. **Food consumption:** Food consumption was not reported.

4. **Plasma analysis:** Rats designated for plasma analysis were dosed twice at 150 mg/kg/day at approximately 24-hour intervals. Blood samples were drawn from the tail vein at 1- and 2-hours post-dosing. The plasma concentration of the test compound was determined by liquid chromotography- mass spectrometry/mass spectrometry (LC-MS/MS).

5. **Preliminary test:** A preliminary test was performed to determine the MTD of the test compound. The MTD was defined as the highest dose that the rats could tolerate without mortality or exhibiting signs of severe clinical toxicity that would necessitate humane euthanasia up to a limit dose of 2000 mg/kg/day. Two rats were dosed twice at approximately 24-hour intervals via oral gavage at a dose level of 500 mg/kg/day. Approximately 2 hours following the second dose, the rats were euthanized and the stomach and duodenum examined macroscopically. If toxicity was observed, the test was to be repeated with a lower dose level.

6. **Comet test:** Rats were dosed at the levels presented in Table 1 two times approximately 24 hours apart; the positive control group was dosed once with aqueous EMS at 200 mg/kg. Approximately two hours after the second dose, the test compound and vehicle control groups were euthanized; the positive control group was euthanized approximately three hours after the single-dose administration. Euthanasia was performed by carbon dioxide asphyxiation. Sections of the liver and duodenum were excised and placed into ice-cold mincing solution (not described) and kept on ice. Single cell suspensions were prepared using tissue-specific methods (additional details were not provided). Comet slides were prepared by first dipping glass slides into 1% normal melting point agarose and allowing them to air dry. The single cell suspensions were diluted (diluent not reported) and mixed with an appropriate volume of 0.5% low melting point agarose. The diluted suspension in agarose was applied (75 µL) to the pre-dipped slide and a cover slip was applied. After the agarose solidified, the cover slips were removed and the slides were placed in lysis buffer (not defined) protected from light at 2-8°C overnight. The following day, the slides were rinsed with electrophoresis buffer (not defined) and placed on a horizontal electrophoresis

unit containing chilled electrophoresis buffer. Additional buffer was added to cover the surfaces of the slides and the slides were allowed to incubate for 20 minutes to allow the cellular DNA to unwind. The slides were then electrophoresed at 18 V with a starting current of approximately 300 mA (0.7-1.0 V/cm) for 30 minutes. It was stated that the buffer temperature was maintained at 4-8°C during unwinding and electrophoresis. After electrophoresis was complete, the slides were removed, rinsed three times for five minutes each with neutralization buffer, and stored refrigerated and protected from light in humidified boxes. The slides were stained with a nucleic acid-specific dye (SYBR GOLD®) and examined with a fluorescence microscope linked to a CCD camera and an image analysis system (Perceptive Instruments COMET IV™).

The slides were first examined for signs of toxicity including background cellular debris and/or increased incidence of excessively-damaged cells (hedgehog cells; see I.B.8 for definition); these cells were excluded from analysis along with cells that had unusual staining artifacts. Fifty cells/slide were scored to yield 150 cells/tissue/rat (Reviewers assume three slides/tissue/rat). The extent of DNA migration (*i.e.*, damage) was measured by determining the % tail intensity defined as the fluorescence detected in the tail that is proportional to the amount of DNA that moved from the head region to the (comet) tail.

7. **Microscopic pathology:** Sections of the duodenum from the vehicle control and test substance-treated groups were fixed in 10% neutral-buffered formalin, routinely processed, stained with hematoxylin and eosin, and examined microscopically for signs of cytotoxicity. The positive control group was not examined microscopically.

## II. RESULTS

**A. PLASMA ANALYSIS:** Plasma analysis data are presented in Table 2. The plasma concentrations of dicamba were 49.1-78.1 mg/L at 1-hour post-dosing and 29.9-43.0 mg/L at 2-hours post-dosing. This confirms the systemic bioavailability of dicamba following oral administration.

**TABLE 2.** Plasma concentration of dicamba in rats administered two doses at 150 mg/kg/day at one- and two-hours post-dosing. [a]

| Time (hours) | Plasma concentration (mg/L) | | |
|---|---|---|---|
| | Rat #407 | Rat #408 | Rat #409 |
| 1 | 78.1 | 65.5 | 49.1 |
| 2 | 40.5 | 29.9 | 43.0 |

a Data were obtained from Table 4 on page 104 of Appendix 5 of MRID 51129101.

## B. PRELIMINARY TEST

1. **Clinical signs of toxicity:** Following administration of the first 500 mg/kg/day dose, both rats were observed with severe clinical signs of toxicity including: flattened posture, underactive behavior, writhing with extension of hindlimbs, uncoordinated movements, rigid body and limbs, red extremities, labored respiration, brown nasal staining, and half-closed eyes; one rat also displayed piloerection. Both rats were euthanized for humane reasons on Day 1.

To determine the MTD, a second group of two rats were administered the test substance at 150 mg/kg/day. Following the first 150 mg/kg/day dose, both rats were noted with the following clinical signs of toxicity: unsteady gait, elevated gait, uncoordinated movements, intermittent rigid body and limbs, flattened posture, underactive behavior, labored respiration, vocalizing on handling, red extremities, hunched posture, and walking on the toes; single rats also displayed writhing with extension of hindlimbs and brown nasal staining. It was stated that these signs were classified as slight or moderate severity with significant recovery after two hours; no clinical signs were reported pre-dosing on Day 2. After administration of the second 150 mg/kg/day dose, unsteady gait, intermittent rigid body and limbs, underactive, hunched posture, high-stepping gait, and waddling gait were seen in both rats; single rats also displayed writhing with extension of hindlimbs and intermittent collapsed posture. Both rats survived until scheduled euthanasia.

2. **Body weights:** Body weight data are presented in Table 3. The rats administered two doses at 150 mg/kg/day exhibited slight body weight losses during the period between the second dose and euthanasia.

**TABLE 3.** Body weights (g) of rats administered two doses of dicamba 24 hours apart. [a]

| Treatment (mg/kg/day) | Rat # | Day 1 | | Day 2 | | Euthanasia | |
|---|---|---|---|---|---|---|---|
| | | Individual | Mean ± SD | Individual | Mean ± SD | Individual | Mean ± SD |
| 500 | 5 | 240 | 242 ± 2.1 | --- | --- | --- | --- |
| | 6 | 243 | | --- | | --- | |
| 150 | 7 | 304 | 292 ± 17.7 | 310 | 298 ± 17.7 | 300 | 290 ± 14.8 |
| | 8 | 279 | | 285 | | 279 | |

a Data were obtained from page 43 in Appendix 1 of MRID 51129101.
--- Rats were euthanized on Day 1.

3. **Necropsy:** The two rats administered 500 mg/kg/day were discarded without further examination on Day 1. At necropsy, the two rats administered 150 mg/kg/day displayed whitened mucosal stomach. Additionally, rat #7 was observed with whitened duodenum and jejunum and stomach devoid of food and containing a clear liquid. The body of this rat was completely rigid within five minutes of death.

The MTD was established at 150 mg/kg/day and the comet test was conducted with dose levels of 37.5, 75, and 150 mg/kg/day.

## C. COMET TEST

1. **Clinical signs of toxicity:** There were no clinical signs of toxicity observed at 37.5 or 75 mg/kg/day or in the positive controls. All rats survived to scheduled euthanasia.

At 150 mg/kg/day, clinical signs included unsteadiness, rigid body and limbs, flattened posture, and a lack of coordination in 6/6 rats following each administration. These signs were not noted prior to administration of the second dose.

2. **Body weights:** Body weight data are presented in Table 4. One 37.5 mg/kg/day rat lost 1 g and one 75 mg/kg/day rat lost 3 g between dosing on Days 1 and 2. These minor losses did not affect the results of the study.

| TABLE 4. Mean (± SD) body weights (g) of rats administered two doses of dicamba 24 hours apart. [a] | | | |
|---|---|---|---|
| **Treatment (mg/kg/day)** | **Day 1** | **Day 2** | **Euthanasia** |
| **0** | 235 ± 13.7 | 237 ± 14.4 | --- |
| **37.5** | 236 ± 12.4 | 240 ± 10.7 | --- |
| **75** | 233 ± 6.0 | 236 ± 6.0 | --- |
| **150** | 233 ± 9.4 | 236 ± 10.8 | --- |
| **EMS [b]** | --- | 231 ± 13.3 | --- |

a Data were obtained from page 44 in Appendix 1 of MRID 51129101. N = 6.
b EMS = 200 mg/kg ethyl methanesulfonate. N = 3.
--- Weights were not recorded.

3. **Necropsy**: There were no effects of treatment noted in the duodenum in the treated rats.

4. **% Tail intensity**: Tail intensity data are presented in Table 5. No effects of treatment were observed on mean tail intensity, mean median tail intensity, or mean number of hedgehog cells in the liver of treated rats. Positive control rats had increased mean tail intensity (not analyzed statistically) and increased mean median tail intensity ($p \leq 0.001$). No hedgehog cells were seen in any group. All values for the liver findings fell within the range of historical control data (presented in an Appendix at the end of this DER).

   In the duodenum, there were large increases in mean tail intensity (not analyzed statistically) and mean median tail intensity ($p \leq 0.001$) at 37.5 and 75 mg/kg/day accompanied by large numbers of hedgehog cells; all values were above the range of historical control data. At 150 mg/kg/day, excessive toxicity, as determined by DNA debris, prevented any cells from being scored. Positive control rats had increased mean tail intensity (not analyzed statistically) and increased mean median tail intensity ($p \leq 0.001$) with no observed hedgehog cells.

| TABLE 5. Tail intensity (%) data in the duodenum and liver of rats administered two doses of dicamba 24 hours apart. [a] | | | | | |
|---|---|---|---|---|---|
| **Treatment** | **Dose (mg/kg/day)** | **# of cells scored** | **Mean (± SD) tail intensity (%)** | **Mean (± SD) median tail intensity (%)** | **Mean (± SD) # Hedgehog cells** |
| | | | **Liver** | | |
| **Vehicle** | **0** | 900 | 2.54 ± 0.2 | 0.53 ± 0.2 | 0 ± 0.0 |
| **Dicamba** | **37.5** | 900 | 2.51 ± 0.2 | 0.49 ± 0.2 | 0 ± 0.0 |
| | **75** | 900 | 2.37 ± 0.1 | 0.39 ± 0.2 | 0 ± 0.0 |
| | **150** | 900 | 2.36 ± 0.3 | 0.50 ± 0.2 | 0 ± 0.0 |
| **EMS** | **200** | 450 | 49.67 ± 3.7 | 50.28 ± 3.1*** | 0 ± 0.0 |
| | | | **Duodenum** | | |
| **Vehicle** | **0** | 900 | 2.85 ± 0.3 | 0.5 ± 0.3 | 0 ± 0.0 |
| **Dicamba** | **37.5** | 900 | 22.96 ± 1.6 | 21.75 ± 2.9*** | 63.2 ± 3.19 [c] |
| | **75** | 900 | 39.23 ± 3.2 | 38.73 ± 3.5*** | 71.5 ± 4.28 [c] |
| | **150** | 0 [b] | --- | --- | --- |
| **EMS** | **200** | 450 | 50.32 ± 3.1 | 50.46 ± 3.6*** | 0 ± 0.0 |

a Data were obtained from Table 1 on page 34 of MRID 51129101.
b Slides were not scored because excessive toxicity (DNA debris) was observed.
c Calculated by the Reviewers from individual data presented in Table 3 on page 41 of MRID 51129101.
*** Significantly different from control; $p \leq 0.001$.
--- No data.

5. **Microscopic pathology**: There were no treatment-related microscopic findings observed in the duodenum of treated animals.

It was stated that extensive ADME studies in the mouse after oral administration have shown that the primary compound excreted is unchanged dicamba. The liver and duodenum both will be exposed to unmetabolized dicamba, and this finding was confirmed by the plasma analysis data. The notable difference in response observed was proposed to be due to local toxicity.

## III. DISCUSSION and CONCLUSIONS

**A. <u>INVESTIGATORS' CONCLUSIONS</u>:** Dicamba has shown evidence of causing an increase in DNA strand breaks in the duodenum of male Crl:CD(SD) rats when administered orally by gavage in this *in vivo* test procedure. However, these increases in DNA strand breaks are directly associated with large increases in hedgehog cells suggesting evidence of cytotoxicity. Therefore, it is unlikely that the DNA strand breaks observed in the duodenum are representative of a true genotoxic response; however, this cannot entirely be ruled out. Dicamba was clearly non-genotoxic in the liver, with bioanalysis of plasma showing that the liver was exposed to Dicamba.

**B. <u>REVIEWER COMMENTS</u>:** The plasma analysis confirmed dicamba was systemically available following two administrations by oral gavage. The preliminary test established an MTD at 150 mg/kg/day. DNA analysis by the comet test demonstrated that there were no effects of treatment on the liver at up to 150 mg/kg/day; EMS induced an expected increase in tail intensity with no increase in hedgehog cells. In the duodenum, the marked increase in tail intensity at 37.5 and 75 mg/kg/day would indicate genotoxicity. However, the marked increase in hedgehog cells observed at these doses, indicating cytotoxicity, are not corroborated by microscopic findings as would be expected. Therefore, the Reviewers consider the results of this study equivocal.

This study is classified as **acceptable / non-guideline**.

**C. <u>STUDY DEFICIENCIES</u>:** The following deficiencies were noted:

- Buffer formulations were not reported.
- Terminal body weights were not recorded.

# Appendix

**Comet Historical Control Data - Liver**

**Wistar Han and Sprague-Dawley Rat**

| Mean % Tail Intensity - Aug-15 – Sep-17 | | | | |
|---|---|---|---|---|
| | Vehicle Control Values | | Positive Control Values | |
| | 57 animals Individual | 10 Studies Group | 38 animals Individual | 10 Studies Group |
| Minimum | 1.3 | 2.1 | 28.7 | 33.5 |
| Maximum | 4.8 | 3.8 | 56.4 | 50.2 |
| Mean | 2.9 | 2.9 | 44.5 | 43.7 |
| Standard Deviation | 0.7 | 0.6 | 6.7 | 6.0 |
| Lower 95% Confidence Limit | 1.5 | 1.7 | 30.9 | 30.2 |
| Upper 95% Confidence Limit | 4.4 | 4.2 | 58.1 | 57.3 |

| Median % Tail Intensity - Aug-15 – Sep-17 | | | | |
|---|---|---|---|---|
| | Vehicle Control Values | | Positive Control Values | |
| | 57 animals Individual | 10 Studies Group | 38 animals Individual | 10 Studies Group |
| Minimum | 0.1 | 0.5 | 26.4 | 30.3 |
| Maximum | 2.6 | 1.1 | 56.0 | 50.0 |
| Mean | 0.6 | 0.6 | 43.7 | 42.8 |
| Standard Deviation | 0.4 | 0.2 | 7.8 | 7.0 |
| Lower 95% Confidence Limit | 0.0 | 0.3 | 28.0 | 26.9 |
| Upper 95% Confidence Limit | 1.5 | 1.0 | 59.5 | 58.6 |

*(copied from page 48 of MRID 51129101)*

**Comet Historical Control Data - Duodenum**

**Wistar Han and Sprague-Dawley Rat**

| **Mean % Tail Intensity - Aug-15 – Sep-17** | | | | |
|---|---|---|---|---|
| | **Vehicle Control Values** | | **Positive Control Values** | |
| | **60 animals Individual** | **10 Studies Group** | **38 animals Individual** | **10 Studies Group** |
| Minimum | 1.2 | 2.3 | 34.9 | 37.8 |
| Maximum | 5.3 | 4.4 | 67.5 | 62.6 |
| Mean | 3.4 | 3.4 | 50.9 | 51.0 |
| Standard Deviation | 0.9 | 0.8 | 8.9 | 7.7 |
| Lower 95% Confidence Limit | 1.6 | 1.7 | 32.8 | 33.5 |
| Upper 95% Confidence Limit | 5.2 | 5.2 | 68.9 | 68.5 |

| **Median % Tail Intensity - Aug-15 – Sep-17** | | | | |
|---|---|---|---|---|
| | **Vehicle Control Values** | | **Positive Control Values** | |
| | **54 animals Individual** | **9 Studies Group** | **33 animals Individual** | **9 Studies Group** |
| Minimum | 0.0 | 0.5 | 32.9 | 36.1 |
| Maximum | 3.0 | 1.3 | 71.5 | 65.2 |
| Mean | 0.9 | 0.9 | 51.2 | 51.2 |
| Standard Deviation | 0.6 | 0.3 | 11.1 | 9.8 |
| Lower 95% Confidence Limit | 0.0 | 0.3 | 28.7 | 28.7 |
| Upper 95% Confidence Limit | 2.2 | 1.5 | 73.8 | 73.8 |

*(copied from page 49 of MRID 51129101)*

# DATA EVALUATION RECORD

DICAMBA

Study Type: OCSPP Non-Guideline; Histopathological Follow-Up Study in Rats

EPA Contract No. EP-W-16-018
Task Assignment No. 34-3-001 (MRID 51129102)

Prepared for
Health Effects Division
Office of Pesticides Program
U.S. Environmental Protection Agency
2777 South Crystal Drive
Arlington, VA 22202

Prepared by
CDM | CSS-Dynamac
Joint Venture
10560 Arrowhead Dr., Suite 500
Fairfax, VA 22030

| | | | |
|---|---|---|---|
| Primary Reviewer: | | Signature: | Michael E. Viana |
| Michael E. Viana, Ph.D. | | Date: | 08/07/2020 |
| Secondary Reviewer: | | Signature: | Sarah Saucier |
| Sarah E. Saucier, Ph.D. | | Date: | 08/21/2020 |
| Quality Assurance: | | Signature: | Scott Studenberg |
| Scott D. Studenberg, Ph.D., DABT | | Date: | 08/26/2020 |
| Project Manager: | | Signature: | Michael E. Viana |
| Michael E. Viana, Ph.D. | | Date: | 08/31/2020 |

This Data Evaluation Record may have been altered by the Health Effects Division subsequent to signing by CDM/CSS-Dynamac Joint Venture personnel. Contractor's role did not include establishing Agency policy.

**EPA Reviewer:** Sarah Dobreniecki **Signature:** Sarah Dobreniecki
**Risk Assessment Branch VII, HED (7509P)** **Date:** 9/16/2020
**Template version 02/06**

# DATA EVALUATION RECORD

**STUDY TYPE:** Histopathological Follow-Up Study in Rats; OCSPP Non-Guideline; OECD 489.

**PC CODE:** 029801

**DP BARCODE:** D458715

**TXR #:** 0058082

**TEST MATERIAL (PURITY):** Dicamba (89.8% a.i.)

**SYNONYMS:** SAN837; Dicamba technical; 3,6-dichloro-2-methoxybenzoic acid

**CITATION:** Herring, T. (2019) Dicamba – Crl:CD(SD) rat histopathological follow-up study. Envigo CRS Ltd., Alconbury, Huntingdon, Cambridgeshire, UK. Laboratory Study No.: NS52VW, February 15, 2019. MRID 51129102. Unpublished.

**SPONSORS:** Syngenta, Ltd., Jealott's Hill International Research Centre, Bracknell, Berkshire, UK

BASF Corp., 100 Park Avenue, Florham Park, NJ

**EXECUTIVE SUMMARY:** In a concurrently-reviewed, non-guideline, *in vivo* comet test (MRID 51129101), increased DNA strand breaks accompanied by increased numbers of hedgehog cells were observed in the duodenum of Crl:CD(SD) male rats administered dicamba in aqueous 0.5% methylcellulose via oral gavage (dose volume 10 mL/kg) at dose levels of 37.5 or 75 mg/kg/day. The present study was performed in order to further investigate the effects on point-of-contact tissues. In this non-guideline, histopathological follow-up study (MRID 51129102), groups of five Crl:CD(SD) male rats were administered dicamba (89.8% a.i., batch # P.MG2726410) in aqueous 0.5% methylcellulose via oral gavage (dose volume 10 mL/kg) at dose levels of 0, 37.5, or 75 mg/kg/day; two doses were administered approximately 24 hours apart. At approximately 2, 6, 24, and 48 hours after the second dose, the rats were euthanized, and sections of the stomach and duodenum were excised, fixed, and routinely processed for microscopic pathological examinations. Sections were visualized with hematoxylin and eosin or by using the terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) and active caspase 3 stains to detect cytotoxicity, necrosis, and/or apoptosis.

There was no treatment-related cytotoxicity, necrosis, or apoptosis at the dose levels administered in this study.

This study is classified as **acceptable / non-guideline**.

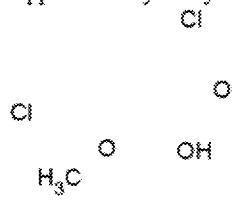
**COMPLIANCE:** Signed and dated Data Confidentiality, GLP Compliance, Flagging, and Quality Assurance statements were provided.

# I. MATERIALS AND METHODS

## A. MATERIALS

### 1. Test material: Dicamba

| | |
|---|---|
| **Description:** | White solid |
| **Batch #:** | P.MG2726410 |
| **Purity:** | 89.8% a.i. |
| **CAS # of TGAI:** | 1918-00-9 |
| **Stability:** | Approximately two years stored at <30°C |
| **Structure:** | |




### 2. Vehicle: Aqueous 0.5% (w/v) methylcellulose (batch #: MKBN2740V; Sigma).

### 3. Test animals

| | |
|---|---|
| **Species:** | Rat (male only) |
| **Strain:** | Crl:CD(SD) |
| **Age / weight at Day 1:** | Approximately 7-8 weeks / 206-266 g |
| **Source:** | Charles River UK Ltd. (Margate, Kent, England) |
| **Housing:** | It was stated the groups were kept in cages; however, additional information (type of cage and rats per cage) were not provided. Rats were provided untreated wood chew blocks and a red plastic shelter for environmental enrichment. |
| **Diet:** | Pelleted Envigo Teklad 2014C diet, *ad libitum* |
| **Water:** | Tap water, *ad libitum* |
| **Environmental conditions** | |
| **Temperature:** | 20-24°C |
| **Humidity:** | 40-70% |
| **Air changes:** | Not provided |
| **Photoperiod:** | 12 hours light/12 hours dark |
| **Acclimation period:** | 5 days minimum |

## B. STUDY DESIGN

**1. In-life dates:** Not reported

**2. Animal assignment:** Following receipt, the rats were weighed and randomly assigned to the groups in Table 1. No additional information was reported.

TABLE 1: Study design [a]

| Group | Dose (mg/kg/day) | Concentration (mg/mL) | Total # males | Time of tissue sampling (hours) | | | |
|---|---|---|---|---|---|---|---|
| | | | | 2 | 6 | 24 | 48 |
| 1 | 0 | 0 | 20 | 5 | 5 | 5 | 5 |
| 2 | 37.5 | 3.75 | 20 | 5 | 5 | 5 | 5 |
| 3 | 75 | 7.5 | 20 | 5 | 5 | 5 | 5 |

a Data were obtained from pages 20 and 52 of MRID 51129102.

3. **Dose-selection rationale:** The doses were the same as those used in the comet test (MRID 51129101).

4. **Preparation of test formulations:** For each dose formulation, an appropriate amount of the test substance (adjusted for purity) was weighed and ground in a mortar with a pestle; vehicle (aqueous 0.5% w/v methylcellulose) was added and mixed to form a paste. Additional vehicle was added to produce a smooth, pourable suspension, and the suspension was brought up to final volume with vehicle and homogenized.

   Homogeneity and stability analyses were reported in MRID 51129101; the results are reported here. Concentration analyses were performed on freshly prepared suspensions; single samples from the top, middle, and bottom (vehicle was sampled twice from the middle) were analyzed.

   **Results**

   **Homogeneity analyses (%CV):** 0.17-4.18%

   **Stability analysis (% of time 0):** 100.7-107.1% following one day storage at ambient temperatures; 101.7-106.6% following eight days storage refrigerated.

   **Concentration analysis (% of nominal):** 96.3-99.3%

   The analytical data indicated that the mixing procedure was adequate and that the variance between nominal and actual dosage to the animals was acceptable.

5. **Dose administration:** The dose suspensions and positive control were administered by oral gavage at a dose volume of 10 mL/kg; the volumes were calculated from the most recent body weights. The dose suspensions were administered twice approximately 24 hours apart.

6. **Statistics:** Statistical analyses were not reported. Because only histopathological results were investigated, the Reviewers do not consider a lack of statistical analyses to be a major deficiency.

## C. METHODS

1. **Observation:** The rats were observed regularly throughout the working day for mortality and morbidity.

2. **Body weight**: All rats were weighed following arrival, on each day of dosing, and at euthanasia. Rats having tissue sampling at 48 hours after the second dose were weighed on Day 3.

3. **Food consumption**: Food consumption was not reported.

4. **Microscopic pathology**: At approximately 2, 6, 24, or 48 hours after administration of the second dose, the rats were euthanized by carbon dioxide asphyxiation. The stomach and duodenum were excised, and sections were fixed in 10% neutral-buffered formalin, routinely processed, and visualized with hematoxylin and eosin or by using the terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) or active caspase 3 stains (methods for the TUNEL and active caspase stains were not reported). All tissues and time points were examined microscopically for signs of cytotoxicity, necrosis, and apoptosis.

## II. RESULTS

A. **CLINICAL SIGNS OF TOXICITY**: There were no clinical signs of toxicity observed in any group. All rats survived to scheduled euthanasia.

B. **BODY WEIGHTS**: Body weight data are presented in Tables 2a-2c. In the 2- and 6-hour sampling groups, all rats in all treatment groups (except one 37.5 mg/kg/day rat; 2-hour sampling time) lost minor amounts of weight. These losses did not affect the results of the study. The 24- and 48-hour sampling groups were unaffected by treatment.

**TABLE 2a.** Mean (± SD) body weights (g) in rats administered two doses of dicamba via oral gavage 24 hours apart and euthanized at 2 and 6 hours after the second dose. [a]

| Dose (mg/kg/day) | Day 1 | Day 2 | Day 2 euthanasia |
|---|---|---|---|
| **2-hour sampling time** | | | |
| **0** | 240 ± 13.8 | 245 ± 13.9 | 242 ± 14.9 |
| **37.5** | 239 ± 12.1 | 243 ± 12.1 | 239 ± 10.6 |
| **75** | 234 ± 17.0 | 239 ± 18.7 | 234 ± 17.9 |
| **6-hour sampling time** | | | |
| **0** | 226 ± 7.1 | 231 ± 7.4 | 222 ± 6.2 |
| **37.5** | 229 ± 6.5 | 234 ± 7.9 | 225 ± 5.5 |
| **75** | 233 ± 22.5 | 235 ± 24.3 | 229 ± 25.8 |

a Data were obtained from Appendix 1 on page 27 of MRID 51129102. N = 5.

**TABLE 2b.** Body weights (g) in rats administered two doses of dicamba via oral gavage 24 hours apart and euthanized at 24 hours after the second dose. [a]

| Dose (mg/kg/day) | Day 1 | Day 2 | Day 3 euthanasia |
|---|---|---|---|
| **0** | 231 ± 14.0 | 237 ± 11.4 | 241 ± 14.0 |
| **37.5** | 245 ± 20.1 | 251 ± 20.5 | 256 ± 21.3 |
| **75** | 233 ± 17.5 | 238 ± 18.8 | 244 ±17.3 |

a Data were obtained from Appendix 1 on page 28 of MRID 51129102. N = 5.

**TABLE 2c.** Body weights (g) in rats administered two doses of dicamba via oral gavage 24 hours apart and euthanized at 48 hours after the second dose. [a]

| Dose (mg/kg/day) | Day 1 | Day 2 | Day 3 | Day 4 euthanasia |
|---|---|---|---|---|
| **0** | 224 ± 11.3 | 230 ± 12.2 | 236 ± 13.1 | 244 ± 12.6 |
| **37.5** | 245 ± 8.7 | 250 ± 6.4 | 259 ± 10.2 | 268 ± 9.6 |
| **75** | 251 ± 11.0 | 257 ± 10.6 | 265 ± 12.4 | 276 ± 12.8 |

a Data were obtained from Appendix 1 on page 29 of MRID 51129102. N = 5.

## C. MICROSCOPIC PATHOLOGY:

1. **Stomach**: It was stated that there was no increase in the number/proportion of apoptotic/necrotic cells in any animal on the hematoxylin and eosin stained sections; further, no lesions of any kind were noted.

   Caspase 3 staining revealed minimal staining of scattered cells in the limiting ridge or glandular stomach in several treated and control rats with no apparent relationship to dose or time after dosing.

   Minimal to slight TUNEL staining was seen in the non-glandular region of the stomach in several treated and control rats. There was no apparent relationship to dose or time after dosing, and these findings were considered artefactual as there was no accompanying caspase 3 staining.

2. **Duodenum**: It was stated that there was no increase in the number/proportion of apoptotic/necrotic cells in any animal on the hematoxylin and eosin stained sections; further, no lesions of any kind were noted.

   It was stated that caspase 3 staining revealed minimal to slight numbers of scattered apoptotic cells throughout the length of the villi in all animals with the number and distribution of these cells having no apparent relationship to dose or time after dosing.

   It was stated that there was no TUNEL staining observed in the duodenum in any animal.

# III. DISCUSSION and CONCLUSIONS

**A. INVESTIGATORS' CONCLUSIONS:** There was no detectable increase in apoptotic/necrotic cells in the stomach or duodenum related to treatment with dicamba.

**B. REVIEWER COMMENTS:** The Reviewers agree with the Investigators' Conclusions. Treatment with dicamba did not cause cytotoxicity, necrosis, or apoptosis at the dose levels administered in this study.

This study is classified as **acceptable / non-guideline**.

**C. STUDY DEFICIENCIES:** The following deficiencies were noted:

- Methods for the TUNEL and caspase 3 staining procedures were not reported.
- Tabular data (including severity grading) for the microscopic findings were not provided.

# DATA EVALUATION RECORD

DICAMBA

Study Type: OCSPP Non-Guideline; Mechanistic Follow-Up Study in Rats

EPA Contract No. EP-W-16-018
Task Assignment No. 34-3-001 (MRID 51129103)

Prepared for
Health Effects Division
Office of Pesticides Program
U.S. Environmental Protection Agency
2777 South Crystal Drive
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Prepared by
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|---|---|---|---|
| Primary Reviewer: | | Signature: | |
| Michael E. Viana, Ph.D. | | Date: | 08/11/2020 |
| Secondary Reviewer: | | Signature: | |
| Sarah E. Saucier, Ph.D. | | Date: | 08/24/2020 |
| Quality Assurance: | | Signature: | |
| Scott D. Studenberg, Ph.D., DABT | | Date: | 08/26/2020 |
| Project Manager: | | Signature: | |
| Michael E. Viana, Ph.D. | | Date: | 08/31/2020 |

This Data Evaluation Record may have been altered by the Health Effects Division subsequent to signing by CDM/CSS-Dynamac Joint Venture personnel. Contractor's role did not include establishing Agency policy.

**EPA Reviewer:** Sarah Dobreniecki **Signature:** Sarah Dobreniecki
**Risk Assessment Branch VII, HED (7509P)** **Date:** 9/16/2020
**Template version 02/06**

# DATA EVALUATION RECORD

**STUDY TYPE:** Mechanistic Follow-Up Study in Rats; OCSPP Non-Guideline; OECD 489.

**PC CODE:** 029801

**DP BARCODE:** D458715

**TXR #:** 0058082

**TEST MATERIAL (PURITY):** Dicamba (89.8% a.i.)

**SYNONYMS:** BAS 183 H; SAN837; 3,6-dichloro-2-methoxybenzoic acid

**CITATION:** Barfield, W. (2020) Dicamba techn. (BAS 183 H; SAN837 techn.): Follow up study to determine potential *ex vivo* effects during comet tissue processing. Covance CRS Ltd., Alconbury, Huntingdon, Cambridgeshire, UK. Laboratory Study No.: MM44NB, March 30, 2020. MRID 51129103. Unpublished.

**SPONSORS:** Syngenta, Ltd., Jealott's Hill International Research Centre, Bracknell, Berkshire, UK

BASF SE, Ludwigshafen, Germany

**EXECUTIVE SUMMARY:** In a concurrently-reviewed, non-guideline, *in vivo* Comet test (MRID 51129101), increased DNA strand breaks accompanied by increased numbers of hedgehog cells were observed in the duodenum of Crl:CD(SD) male rats administered dicamba in aqueous 0.5% methylcellulose via oral gavage (dose volume 10 mL/kg) at dose levels of 37.5 or 75 mg/kg/day. In order to further investigate potential *ex vivo* effects on point-of-contact tissues, the present study was performed. In this non-guideline, mechanistic follow-up study (MRID 51129103), groups of three Crl:CD(SD) male rats were administered dicamba (89.8% a.i., batch # P.MG2726410) in aqueous 0.5% methylcellulose via oral gavage (dose volume 10 mL/kg) at dose levels of 0 or 75 mg/kg/day; two doses were administered approximately 24 hours apart. At approximately 0.5, 1, 2, 4, or 6 hours after the second dose, the rats were euthanized; control rats were euthanized 2 hours after the second administration of vehicle. A seventh group of three rats were administered three doses at 25 mg/kg at 30-minute intervals; this dosing was performed twice 24 hours apart, and the rats were euthanized 2 hours after the second dosing was completed. A positive control group was administered ethyl methanesulfonate in purified water at 200 mg/kg and euthanized 3 hours after the single dose. Single cell suspensions of the duodenum and liver were prepared for comet analysis.

Comet test data showed increased % tail intensity accompanied by increased hedgehog cells across some, but not all, time points. The positive control generated data consistent with expected findings. Intracellular pH data was subjective, not quantifiable, and inconclusive.

Mincing solution pH and osmolality was not affected by incubation times or cell suspension preparation. There were no microscopic findings of cytotoxicity, necrosis, or apoptosis.

This study is classified as **acceptable / non-guideline**.

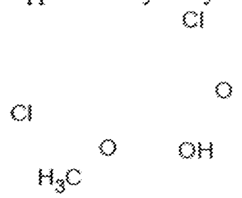
**COMPLIANCE:** Signed and dated Data Confidentiality, GLP Compliance, Flagging, and Quality Assurance statements were provided.

# I. MATERIALS AND METHODS

## A. MATERIALS

**1. Test material:** Dicamba

| | |
|---|---|
| **Description:** | White solid |
| **Batch #:** | P.MG2726410 |
| **Purity:** | 89.8% a.i. |
| **CAS # of TGAI:** | 1918-00-9 |
| **Stability:** | Approximately two years stored at <30°C |
| **Structure:** | |




**2. Vehicle:** Aqueous 0.5% (w/v) methylcellulose (batch #: SLBR8963V; Sigma).

**3. Test animals**

| | |
|---|---|
| **Species:** | Rat (male only) |
| **Strain:** | Crl:CD(SD) |
| **Age / weight at Day 1:** | Approximately 7-8 weeks / 218-250 g |
| **Source:** | Charles River UK Ltd. (Margate, Kent, England) |
| **Housing:** | Three rats/cage; however, additional information (type of cage) were not provided. Rats were provided untreated wood chew blocks and a red plastic shelter for environmental enrichment. |
| **Diet:** | Pelleted Envigo Teklad 2014C diet, *ad libitum* |
| **Water:** | Tap water, *ad libitum* |
| **Environmental conditions** | |
| **Temperature:** | 20-24°C |
| **Humidity:** | 40-70% |
| **Air changes:** | Not provided |
| **Photoperiod:** | 12 hours light/12 hours dark |
| **Acclimation period:** | 5 days minimum |

## B. STUDY DESIGN

**1. In-life dates:** Not reported

**2. Animal assignment:** Following receipt, the rats were weighed and randomly assigned to the groups in Table 1. No additional information was reported.

| TABLE 1: Study design [a] | | | | | |
|---|---|---|---|---|---|
| Group | Treatment | Concentration (mg/mL) | Dose (mg/kg/day) | Time of tissue sampling (hours) [b] | # of rats |
| 1 | Vehicle | 0 | 0 | 2 | 3 |
| 2 | Dicamba | 7.5 | 75 | 0.5 | 3 |
| 3 | Dicamba | 7.5 | 75 | 1 | 3 |
| 4 | Dicamba | 7.5 | 75 | 2 | 3 |
| 5 | Dicamba | 7.5 | 75 | 4 | 3 |
| 6 | Dicamba | 7.5 | 75 | 6 | 3 |
| 7 | Dicamba | 2.5 [c] | 75 | 2 | 3 |
| 8 | EMS [d] | 20 | 200 | 3 | 3 |

a Data were obtained from page 20 of MRID 51129103.
b Time after the second or final dose on Day 2.
c Rats were administered three doses of 25 mg/kg at 30-minute intervals on two consecutive days.
d EMS = ethyl methanesulfonate, single 200 mg/kg dose.

3. **Dose-selection rationale:** The 75 mg/kg/day dose was used in the initial comet test (MRID 51129101).

4. **Preparation of test formulations:** For each dose formulation, an appropriate amount of the test substance (adjusted for purity) was weighed and ground in a mortar with a pestle; vehicle (aqueous 0.5% w/v methylcellulose) was added and mixed to form a paste. Additional vehicle was added to produce a smooth, pourable suspension, and the suspension was brought up to final volume with vehicle and homogenized.

   Homogeneity and stability analyses were reported in MRID 51129101; the results are reported here. Concentration analyses were not reported. It was stated that the pH of the 2.5 mg/mL formulation was 2.49 and the pH of the 7.5 mg/mL formulation was 2.10.

5. **Dose administration:** The dose suspensions and positive control were administered by oral gavage at a dose volume of 10 mL/kg; the volumes were calculated from the most recent body weights. The dose suspensions were administered twice approximately 24 hours apart.

6. **Statistics:** Statistical analyses were not reported. The data generated were compared to historical control data.

## C. METHODS

1. **Observation:** The rats were observed regularly throughout the working day for mortality and morbidity.

2. **Body weight:** All rats were weighed following arrival, on each day of dosing, and at euthanasia.

3. **Food consumption:** Food consumption was not reported.

4. **Euthanasia:** The rats were euthanized by carbon dioxide asphyxiation at the times indicated in Table 1 (after the second or final dose on Day 2).

5. **Single cell preparations:** Two methods for preparation of single cell suspensions from the duodenum samples were used. Comet slides were prepared from the duodenum of animals in Groups 1 to 8 by using Method 1 and Groups 1 to 6 by using Method 2. Single cell suspensions of liver samples also were prepared and used for intracellular pH measurements.

a. **Duodenum method 1:** The duodenum was excised, cleaned with complete mincing solution (not described) by using the back of a scalpel blade, and placed into a tube with additional mincing solution. The samples were incubated on ice for approximately 30 minutes (± 10 minutes) and cleaned a second time as above. The tissue was transferred to a dish, additional complete mincing solution was added, and the tissue was minced by using a scalpel blade to release the cells. The cells were stored in a tube on ice until slide preparation.

b. **Duodenum method 2:** The duodenum was excised, placed in ice-cold Merchants solution (not described), and vortexed for 15 seconds. The tissue was moved to a petri dish and the inner surface scraped twice with the back of a scalpel blade to remove any waste material. The tissue was moved to fresh ice-cold Merchants solution, vortexed for 15 seconds, and placed back in the dish. Ice-cold Merchants solution (150 µL) was pipetted onto each duodenum and the inner surface scraped three times (the Reviewers assume this step released the cells). The cell suspension was stored in a tube.

c. **Liver suspensions:** Small sections of liver (approximately 0.5 $cm^3$) were cut and washed in fresh complete mincing solution (not described) to remove as much blood as possible. Fresh mincing solution was added, and the liver section was cut into several smaller pieces. The pieces were transferred to a portion of 150-µm bolting cloth (not described) placed over a tube and the liver was pushed through the cloth. Additional mincing solution was added and any remaining liver was pushed through the cloth. The cell suspension was stored on ice until slide preparation.

6. **Intracellular pH measurements:** Intracellular pH was measured with two commercially-available kits (Fluorometric Intracellular pH Assay Kit, Sigma [kit 1] and pHrodo™ Green AM intracellular pH Indicator, ThermoFischer [kit 2]). Briefly, the remaining cell preparations obtained from the duodenum by using Method 1 were divided into two equal portions and centrifuged. Two portions (500 µL) of the liver cell preparations were centrifuged and the supernatant discarded. For kit 1, the cell pellets were stained with the dye reagent (100 µL) and incubated for 30 minutes at 37°C in a 5% $CO_2$ atmosphere. The cells were qualitatively assessed for pH with a fluorescence microscope (λ excitation = 490 nm / λ emission = 535 nm). It was stated that the dye used (BCFL-AM; not described) penetrated into the cells. An acetoxymethyl (AM) group was cleaved by intracellular esterase hydrolysis and the resulting BCFL fluorophore was retained in the cell. The fluorescence intensity of the dye peaks at neutral pH and is reduced by lowering the pH. Thus, a reduction of the fluorescence is a signal for low intracellular pH values. For kit 2, the cell pellets were washed once in LCIS buffer (not described) and centrifuged again. The dye reagent (100 µL) was added to the pellet, the pellet was resuspended, and the suspension was incubated at 34-39°C for 30 minutes. The cells were then qualitatively

assessed for pH with a fluorescence microscope (λ excitation = 490 nm / λ emission = 535 nm). The dye used for this stain is weakly fluorescent at neutral pH but increasingly fluorescent as the pH drops.

7. **Mincing solution pH and osmolality measurements:** The pH and osmolality of the mincing solution were determined pre-incubation, post-incubation, and after the cell suspensions were prepared from Groups 1 to 7.

8. **Dicamba concentration analysis:** The supernatant of the intracellular pH aliquots from the duodenum preparations were stored at −30°C to −10°C. In addition to this, the mincing buffer remaining after the 30 minutes incubations also was stored at −30°C to −10°C. It was stated that the concentration analyses would be performed under a separate study.

9. **Comet test:** Single cell suspensions were prepared as described above. Comet slides were prepared by first dipping glass slides into 1% normal melting point agarose and allowing them to air dry. The single cell suspensions were diluted (diluent not reported) and mixed with an appropriate volume of 0.5% low melting point agarose. The diluted suspension in agarose was applied (75 µL) to the pre-dipped slide and a cover slip was applied. After the agarose solidified, the cover slips were removed. For cell suspensions prepared by Method 2, the slides were placed in lysis buffer (not defined) protected from light at 2-8°C overnight. For cell suspensions prepared by Method 1, the slides were placed in lysis buffer one hour after preparation of the first slide and refrigerated overnight. The following day, the slides were rinsed with electrophoresis buffer (not defined) and placed on a horizontal electrophoresis unit containing chilled electrophoresis buffer. Additional buffer was added to cover the surfaces of the slides and the slides were allowed to incubate for 20 minutes to allow the cellular DNA to unwind. The slides were electrophoresed at 18 V with a starting current of approximately 300 mA (0.7-1.0 V/cm) for 30 minutes. It was stated that the buffer temperature was maintained at 4-9°C during unwinding and electrophoresis. When electrophoresis was complete, the slides were removed, rinsed three times for five minutes each with neutralization buffer, and stored refrigerated and protected from light in humidified boxes. The slides were stained with a nucleic acid-specific dye (SYBR GOLD®) and examined with a fluorescence microscope linked to a CCD camera and image analysis system (Perceptive Instruments COMET IV™).

   The slides were first examined for signs of toxicity including background cellular debris and/or increased incidence of excessively damaged cells (hedgehog cells); these cells were excluded from analysis along with cells that had unusual staining artefacts. Fifty cells/slide were scored to yield 150 cells/tissue/rat (Reviewers assume three slides/tissue/rat). The extent of DNA migration (*i.e.*, damage) was measured by determining the % tail intensity defined as the fluorescence detected in the tail that is proportional to the amount of DNA that moved from the head region to the (comet) tail.

10. **Microscopic pathology:** Duodenum sections were incubated in ice-cold mincing solution for 30 minutes (as in Method 1), then fixed in 10% buffered formalin, routinely processed, and stained with either hematoxylin and eosin or active caspase 3. Slides from Groups 1-7 were examined microscopically for cytotoxicity, necrosis, or apoptosis.

## II. RESULTS

**A. CLINICAL SIGNS OF TOXICITY:** There were no clinical signs of toxicity observed in any group. All rats survived to scheduled euthanasia.

**B. BODY WEIGHTS:** Body weight data are presented in Table 2. All rats in all groups lost minor amounts of weight (−4 g to −11 g) between administration of the second dose and euthanasia. These losses did not affect the results of the study.

**TABLE 2.** Mean (± SD) body weights (g) in rats administered two 75 mg/kg/day doses of dicamba via oral gavage 24 hours apart. [a]

| Dose (mg/kg/day) | Time of euthanasia (hours post-dose) [b] | Day 1 | Day 2 | Day 2 euthanasia |
|---|---|---|---|---|
| **0** | 2 | 232 ± 6.2 | 239 ± 5.1 | 235 ± 5.5 |
| **75** | 0.5 | 233 ± 9.3 | 238 ± 12.5 | 233 ± 12.4 |
| **75** | 1 | 236 ± 6.7 | 242 ± 6.7 | 235 ± 6.4 |
| **75** | 2 | 231 ± 15.5 | 235 ± 14.6 | 231 ± 14.4 |
| **75** | 4 | 234 ± 13.0 | 237 ± 11.6 | 233 ± 11.5 |
| **75** | 6 | 243 ± 11.3 | 250 ± 12.3 | 241 ± 10.6 |
| **75 [c]** | 2 | 226 ± 4.0 | 228 ± 4.2 | 223 ± 5.1 |
| **EMS 200 [d]** | 3 | --- | 251 ± 3.5 | 240 ± 3.2 |

a Data were obtained from Appendix 1 on page 48 of MRID 51129103. N = 3.
b Time after the second or final dose on Day 2.
c Rats were administered three doses of 25 mg/kg at 30-minute intervals on two consecutive days.
d EMS = ethyl methanesulfonate, single 200 mg/kg dose.
--- No data.

**C. COMET TEST:** Percentage tail intensity and hedgehog cell data are presented in Tables 3 and 4.

### 1. % Tail intensity

**a. Method 1:** Tissue samples obtained at the 0.5-, 4-, and 6-hour time points from the rats administered two 75 mg/kg/day doses, and tissue samples obtained at 2 hours from the rats administered three 25 mg/kg doses all displayed increased mean and mean median % tail intensity that was greater than the 95% confidence limits of the historical control data. Tissue samples obtained at the 1- and 2-hour time points were similar to control. Positive control samples displayed marked increases in mean and mean median % tail intensity.

**b. Method 2:** Tissue samples from all time points from all treated rats displayed increased mean and mean median % tail intensity that was greater than the 95% confidence limits of the historical control data. Positive control samples were not collected by using method 2.

| **TABLE 3.** Mean (± SD) % tail intensity and median % tail intensity in rats administered two 75 mg/kg/day doses of dicamba via oral gavage 24 hours apart. [a] | | | | | |
|---|---|---|---|---|---|
| **Dose (mg/kg/day)** | **Time of euthanasia (hours post-dose)** | **% intensity** | | **Median % intensity** | |
| | | **Method 1** | **Method 2** | **Method 1** | **Method 2** |
| **0** | 2 | 2.23 ± 0.5 | 2.57 ± 0.2 | 0.35 ± 0.1 | 0.9 ± 0.1 |
| **75** | .5 | **15.19 ± 0.3** | **6.51 ± 0.9** | **11.29 ± 0.9** | **3.08 ± 1.1** |
| **75** | 1 | 3.69 ± 0.4 | **7.72 ± 1.7** | 0.98 ± 0.6 | **4.10 ± 1.3** |
| **75** | 2 | 3.03 ± 0.6 | **8.36 ± 0.8** | 0.77 ± 0.3 | **3.59 ± 1.4** |
| **75** | 4 | **11.58 ± 1.8** | **8.79 ± 4.2** | **7.08 ± 1.5** | **5.01 ± 2.5** |
| **75** | 6 | **15.68 ± 4.6** | **7.54 ± 4.2** | **11.68 ± 5.0** | **3.83 ± 2.8** |
| **75 [b]** | 2 | **10.47 ± 3.7** | --- | **6.08 ± 3.8** | --- |
| **EMS 200 [c]** | 3 | **36.58 ± 8.3** | --- | **33.26 ± 10.0** | --- |

a Data were obtained from Table 1 on page 34 of MRID 51129103. N = 3.
b Rats were administered three doses of 25 mg/kg at 30-minute intervals on two consecutive days.
c EMS = ethyl methanesulfonate, single 200 mg/kg dose.
**Bold values** are >95% control limit of laboratory historical control database.
--- No data.

## 2. Hedgehog cells

**a. Method 1:** Tissue samples obtained at the 0.5-, 4-, and 6-hour time points from the rats administered two 75 mg/kg/day doses, and tissue samples obtained at 2 hours from the rats administered three 25 mg/kg/day doses all displayed markedly increased numbers of hedgehog cells. Tissue samples obtained at the 1-hour time point displayed a slight increase in hedgehog cells. Tissue samples from the 2-hour time point, control, and positive control groups had no hedgehog cells.

**b. Method 2:** Tissue samples from all time points from all treated rats displayed increased numbers of hedgehog cells; however, hedgehog cells also were observed in the vehicle control group. Positive control samples were not collected by using method 2.

| **TABLE 4.** Mean (± SD) hedgehog cell data in rats administered two 75 mg/kg/day doses of dicamba via oral gavage 24 hours apart. [a] | | | |
|---|---|---|---|
| **Dose (mg/kg/day)** | **Time of euthanasia (hours post-dose)** | **# of Hedgehog cells** | |
| | | **Method 1** | **Method 2** |
| **0** | 2 | 0.0 ± 0.0 | 8.3 ± 1.2 |
| **75** | .5 | 35.3 ± 8.5 | 10.3 ± 4.5 |
| **75** | 1 | 1.3 ± 1.5 | 14.0 ± 2.6 |
| **75** | 2 | 0.0 ± 0.0 | 11.0 ± 2.0 |
| **75** | 4 | 39.3 ± 6.7 | 15.7 ± 11.6 |
| **75** | 6 | 50.7 ± 9.7 | 16.0 ± 10.1 |
| **75 [b]** | 2 | 20.7 ± 14.3 | --- |
| **EMS 200 [c]** | 3 | 0.0 ± 0.0 | --- |

a Data were obtained from Table 3 on page 39 of MRID 51129103. N = 3.
b Rats were administered 3 doses of 25 mg/kg at 30-minute intervals on two consecutive days.
c EMS = ethyl methanesulfonate, single 200 mg/kg dose.
--- No data.

**3. Intracellular pH:** It was stated that the differences in intracellular pH detected by using kit 1 were not substantial but that an impact on intracellular pH levels could be detected. The Reviewers do not consider this data useful to make any conclusions regarding intracellular pH. Responses were described as no fluorescence, poor quality, low intensity, or slight intensity; quantification of these descriptions was not possible.

4. **Mincing solution pH and osmolality measurements:** Measurements of pH and osmolality of the mincing solution post-incubation or following preparation of the cell suspensions were generally similar to the values obtained pre-incubation.

5. **Microscopic pathology:** There were no effects of treatment noted during the microscopic examinations.

## III. DISCUSSION and CONCLUSIONS

A. **INVESTIGATORS' CONCLUSIONS:** This study confirmed previous data, that Dicamba techn. (BAS 183 H; SAN837 techn.) induces DNA damage under the described circumstances. However, it remains unclear whether this damage is caused by a direct interaction with the host DNA or is a product of indirect effects such as exposure to low intracellular pH. Several parameters such as the intracellular pH determination, the length of the exposure periods and the split dosing suggest the latter mechanism. However, the data are too inconclusive for a definitive outcome.

B. **REVIEWER COMMENTS:** The Reviewers agree with the Investigators' Conclusions that the data generated in the present study are not conclusive.

Comet test data showed increased % tail intensity accompanied by increased hedgehog cells across some, but not all, time points. The positive control generated data consistent with expected findings. Intracellular pH data was subjective, not quantifiable, and inconclusive. Mincing solution pH and osmolality were not affected by incubation times or cell suspension preparation. There were no microscopic findings of cytotoxicity, necrosis, or apoptosis.

This study is classified as **acceptable / non-guideline**.

C. **STUDY DEFICIENCIES:** The following deficiencies were noted:

- Concentration analysis of the formulations were not reported.
- Methods for caspase 3 staining procedures were not reported.

# DATA EVALUATION RECORD

DICAMBA

Study Type: OCSPP Non-Guideline; [$^{14}$C]-Dicamba: Duodenum Kinetics in Rats

EPA Contract No. EP-W-16-018
Task Assignment No. 34-3-001 (MRID 51129104)

Prepared for
Health Effects Division
Office of Pesticides Program
U.S. Environmental Protection Agency
2777 South Crystal Drive
Arlington, VA 22202

Prepared by
CDM | CSS-Dynamac
Joint Venture
10560 Arrowhead Dr., Suite 500
Fairfax, VA 22030

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|---|---|---|---|
| Primary Reviewer: | | Signature: | |
| Michael E. Viana, Ph.D. | | Date: | 08/14/2020 |
| Secondary Reviewer: | | Signature: | |
| Sarah E. Saucier, Ph.D. | | Date: | 08/25/2020 |
| Quality Assurance: | | Signature: | |
| Scott D. Studenberg, Ph.D., DABT | | Date: | 08/27/2020 |
| Project Manager: | | Signature: | |
| Michael E. Viana, Ph.D. | | Date: | 08/31/2020 |

This Data Evaluation Record may have been altered by the Health Effects Division subsequent to signing by CDM/CSS-Dynamac Joint Venture personnel. **Contractor's role did not include establishing Agency policy.**

**EPA Reviewer:** Sarah Dobreniecki **Signature:** Sarah Dobreniecki
**Risk Assessment Branch VII, HED (7509P)** **Date:** 9/16/2020
Template version 02/06

# DATA EVALUATION RECORD

**STUDY TYPE:** Mechanistic Follow-Up Study in Rats; OCSPP Non-Guideline; OECD 489.

**PC CODE:** 029801

**DP BARCODE:** D458715

**TXR #:** 0058082

**TEST MATERIAL (RADIOCHEMICAL PURITY):** [$^{14}$C]-Dicamba (99% a.i.)

**SYNONYMS:** BAS 183 H; SAN837 technical; 3,6-dichloro-2-methoxybenzoic acid

**CITATION:** Hilton, A. (2020) [$^{14}$C]-Dicamba: duodenum kinetics in rats. Covance CRS Ltd., Alconbury, Huntingdon, Cambridgeshire, UK. Laboratory Study No.: MT42NJ, February 28, 2020. MRID 51129104. Unpublished.

**SPONSORS:** Syngenta, Ltd., Jealott's Hill International Research Centre, Bracknell, Berkshire, UK

BASF SE, Carl-Bosch-Strasse 38, Ludwigshafen am Rhein, Germany

**EXECUTIVE SUMMARY:** In a concurrently-reviewed, non-guideline, *in vivo* comet test (MRID 51129101), increased DNA strand breaks accompanied by increased numbers of hedgehog cells were observed in the duodenum of Crl:CD(SD) male rats administered dicamba in aqueous 0.5% methylcellulose via oral gavage (dose volume 10 mL/kg) at dose levels of 37.5 or 75 mg/kg/day. The present study was performed to obtain the absorption kinetics and rates and routes of excretion of dicamba following oral administration to rats. In this non-guideline, absorption kinetics follow-up study (MRID 51129104), groups of four Crl:CD(SD) male rats were administered [$^{14}$C]-dicamba (radiochemical purity 99%, batch # WJE-I-57) in aqueous 0.5% methylcellulose via oral gavage (dose volume 10 mL/kg) at a dose level of 75 mg/kg/day; two doses were administered approximately 24 hours apart. At approximately 0.5, 1, 2, 4, or 6 hours after the second dose, the rats were euthanized. Whole blood and plasma, duodenum sections, mincing solutions and scrapings, liver sections, and urine samples were obtained at each time point and analyzed for radioactivity.

No clinical signs of toxicity were reported.

[$^{14}$C]-Dicamba is rapidly absorbed after oral gavage administration. The maximum concentration of [$^{14}$C]-dicamba in whole blood was observed at 0.5 hours after the second dose and declined steadily to the final 6-hour sample. Similarly, the maximum concentration of [$^{14}$C]-dicamba in plasma was observed at 0.5 hours after the second dose and declined steadily to

the final 6-hour sample. Plasma concentrations were consistently greater than those in whole blood.

In the duodenum sections, mean total radioactivity and mean concentration of radioactivity were greatest at 0.5 hours after the second dose and declined with time. Concentrations were still measurable at 6 hours after the second dose. Concentrations in duodenum sections were greatest in section A (immediately after the stomach) and declined from sections A to B and generally declined from sections B to C between 0.5-2 hours after the second dose. At 4 and 6 hours, mean concentrations were generally similar across all sections. Mean total radioactivity and mean concentrations of radioactivity in the mincing solutions and scrapings were less than those found in the duodenum sections and followed the same time course, approaching the limit of quantitation at 6 hours. Mean concentrations of radioactivity in the liver sections were greatest at 0.5 hours after the second dose and declined with time to 6 hours.

Radioactivity was detectable in the urine at 1 hour following the first dose with the greatest concentration at 4 hours. Radioactivity concentrations fell to the lowest concentration at 24 hours after the first dose. Following the second dose, radioactivity began to rise, with the greatest concentration at 2 hours after the second dose and falling with time to the final collection at 6 hours.

This study is classified as **acceptable / non-guideline**.

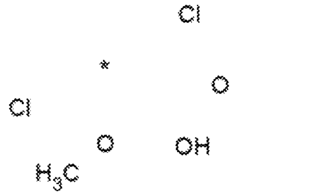
**COMPLIANCE:** Signed and dated Data Confidentiality, GLP Compliance, Flagging, and Quality Assurance statements were provided.

# I. MATERIALS AND METHODS

## A. MATERIALS

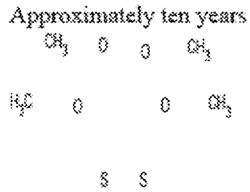
### 1. Test compounds:

**Radiolabeled test compound:** [Phenyl-U-$^{14}$C]-SAN837H (dicamba)
**Radiochemical purity:** 99% (TLC)
**Specific activity:** 182.5 µCi/mg (6.75 MBq/mg)
**Batch #:** WJE-I-57
**Expiration/storage:** February 29, 2020 / ≤ −80°C
**Structure:**




* indicates position of $^{14}$C-label

**Non-Radiolabeled TGAI:** Dicamba
**Description:** White solid
**Lot #:** P.MG2726410
**Purity:** 89.8% a.i.
**CAS # of TGAI:** 1918-00-9
**Expiration/storage:** Approximately ten years stored at <30°C
**Structure:**



### 2. Vehicle: Aqueous 0.5% (w/v) methylcellulose (batch # and source not provided).

### 3. Test animals

**Species:** Rat (male only)
**Strain:** Sprague Dawley [Crl:CD(SD)]
**Age / weight at Day 1:** Approximately 1-2 months / 220-256 g
**Source:** Charles River UK Ltd. (Margate, Kent, England)
**Housing:** During acclimation, rats were housed up to 5/cage in solid-bottom polycarbonate cages with stainless steel lids and wood-flake bedding. Environmental enrichment (wooden chew block and plastic shelter) were provided. After dosing, rats designated for excretion phases were housed individually in glass metabolism cages; rats in the other sub-groups were returned to their battery cages.
**Diet:** VRF1 diet, *ad libitum*.
**Water:** Tap water, *ad libitum*.
**Environmental conditions**
**Temperature:** 20-24°C
**Humidity:** 40-70%
**Air changes:** Not provided
**Photoperiod:** 12 hours light/12 hours dark
**Acclimation period:** 4-5 days minimum

### 4. Preparation of dosing solutions: It was stated that method suitability and formulation homogeneity were established by using a trial dose prior to dose formulation (no further

details provided). A stock solution of [$^{14}$C]-dicamba was prepared by dissolving the radiolabel in acetonitrile (0.5 mL) and mixing by inversion until dissolved. The stock solution was stored at −70°C ± 10°C. The dose formulations were prepared by first combining [$^{14}$C]-dicamba and non-labeled dicamba in acetonitrile to yield the desired specific activity. This dilution was placed in a mortar and dried under a stream of nitrogen. The vehicle (aqueous 0.5% [w/v] methylcellulose) was added and the radiolabel suspended with a mortar. This suspension was transferred to a dose vessel (not described), stirred continuously, and sonicated. Formulation details are presented in Table 1.

**TABLE 1.** Dose preparations. [a]

| Parameter | Day 1 | Day 2 |
|---|---|---|
| Weight [$^{14}$C]-dicamba (mg) | 5.018 | 4.908 |
| Weight non-labeled dicamba (mg) | 524.76 | 531.941 |
| Volume of vehicle (mL) | 70 | 70 |
| Calculated specific activity (dpm/mg) | 3867430 | 3791451 |
| Concentration of [$^{14}$C]-dicamba (mg/mL) | 7.497 | 7.599 |
| Volume administered (mL/kg) | 10 | 10 |

a Data obtained from Appendix 2 on pages 44-45 of MRID 51129104.

Triplicate portions of each formulation were sampled pre- and post-dosing and assayed by liquid scintillation counting (LSC).

## B. STUDY DESIGN AND METHODS

**1. In-life dates:** Not reported.

**2. Animal assignment:** Study details are presented in Table 2. Following receipt, the rats were weighed and randomly assigned to the groups. No additional information was reported.

**TABLE 2.** Absorption kinetics and excretion study after administration of [$^{14}$C]-dicamba by two oral gavage doses of 75 mg/kg/day to rats. [a]

| Group | Number of Rats | Sampling time (hours) | Body weight (g) [b] | | Dose administered (mg) [b] | | Dose administered (mg/kg/day) [b] | |
|---|---|---|---|---|---|---|---|---|
| | | | Day 1 | Day 2 | Day 1 | Day 2 | Day 1 | Day 2 |
| 1 | 4 | 0.5 | 228 ± 6 | 235 ± 8 | 17.05 ± 0.48 | 17.95 ± 0.65 | 74.9 ± 0.4 | 76.3 ± 0.3 |
| 2 | 4 | 1 | 238 ± 10 | 247 ± 7 | 17.80 ± 0.89 | 18.71 ± 0.36 | 74.8 ± 0.6 | 75.9 ± 0.7 |
| 3 | 4 | 2 | 241 ± 9 | 247 ± 9 | 17.99 ± 0.53 | 18.71 ± 0.57 | 74.7 ± 0.6 | 75.9 ± 0.7 |
| 4 | 4 | 4 | 248 ± 5 | 256 ± 5 | 18.55 ± 0.37 | 19.57 ± 0.38 | 74.8 ± 0.4 | 76.5 ± 0.7 |
| 5 | 4 | 6 | 240 ± 11 | 248 ± 11 | 17.99 ± 1.02 | 18.81 ± 0.66 | 75.1 ± 0.8 | 75.9 ± 0.9 |

a Data were obtained from page 17 and Appendix 2 on pages 44-45 of MRID 51129104.
b Mean ± SD calculated by the Reviewers.

### 3. Dosing and sample collection

**a. Dose selection:** The 75 mg/kg/day dose level was used in previously performed comet test (MRID 51129401).

**b. Dosing:** The dose suspensions were administered by oral gavage at a dose volume of 10 mL/kg; the volumes were calculated from the Day 1 and 2 body weights. The dose suspensions were administered twice approximately 24 hours apart. The mean (± SD) doses administered for each group are shown in Table 1.

All rats were observed immediately after dosing, at approximately 1-2 hours post-dosing, and at least one other time during the day for clinical signs of toxicity.

c. **Sample collection:** At the times specified in Table 2, the rats were anesthetized with isoflurane and blood samples (approximately 8 mL) were obtained by cardiac puncture. Duplicate portions (50 µL) were removed for radioanalysis and the remaining portion was centrifuged to yield plasma. Duplicate portions (50 µL) of plasma were removed for radioanalysis. The rats were euthanized by cervical dislocation and the duodenum, remaining gastrointestinal tract (including contents), and liver were excised; the residual carcass was retained but not analyzed. The duodenum was isolated and three 2-cm lengths were cut starting from the stomach. The sections were rinsed with mincing solution (not described) and scraped clean; each section was then placed in a separate scintillation vial.

Urine and feces were collected separately from the rats of Group 5 (6-hour sampling time) only. Urine was collected from each rat at 0-1, 1-2, 2-4, 4-6, and 6-24 hours after the first dose and 0-1, 1-2, 2-4, and 4-6 hours after the second dose. Feces were collected during 0-24 hours after the first dose and 0-6 hours after the second dose; the cages were washed with water (100 mL) at each feces collection. The urine and feces containers were cooled with dry ice.

All samples were stored at −20°C ± 10°C until analysis.

4. **Sample treatment:** Treatment of the fecal samples was not discussed and fecal results were not reported.

a. **Urine:** The urine samples were thawed and weighed, and duplicate weighed portions were added directly to scintillation solvent.

b. **Duodenum:** The duodenum sections were weighed and digested with a tissue solubilizer (Goldisol) at approximately 55°C until the digestion was complete. The digest was mixed with scintillation solvent.

c. **Mincing solution:** The mincing solution from each duodenum section was weighed and solubilized in a mixture of water, methanol, and Triton X-405 (6:3:1; v/v) with 80 g/L sodium hydroxide at approximately 55°C until the digestion was complete. The digest was weighed, and duplicate weighed portions were added directly to scintillation solvent.

d. **Liver:** The liver samples were thawed, weighed, and mechanically homogenized. Duplicate weighed portions were digested with tissue solubilizer as for duodenum sections. The digest was mixed with scintillation solvent.

e. **Blood and plasma:** Duplicate weighed portions of blood and plasma were removed for radioanalysis; plasma was added directly to scintillation solvent and blood was combusted.

## 5. Analytical methodology

**a. Combustion analyses:** Blood samples were combusted with a sample oxidizer. Combustion products were trapped with a commercially-available absorbent and mixed with scintillation solvent. Recovery tests were conducted by combusting a commercial standard fortified with appropriate radioactivity, and efficiency was generally >95%. Radioactivity measurements were corrected for combustion efficiency.

**b. Liquid scintillation counting (LSC):** Radioactivity was measured using LSC having automatic quench correction (additional details not provided). Background levels were determined and radioactivity less than twice background was considered below the limit of quantification.

**c. Thin-layer chromatography (TLC):** Radiochemical purities of the stock solution and dose formulations were determined by TLC. TLC was conducted with commercially-available normal phase plates (silica gel 60 $F_{254}$, 0.25 mm thickness) with a toluene:acetone:acetic acid (65:35:5, v:v) solvent system. Linear-scaled radiochromatograms were visualized with an image analyzer (FLA-5000; Fuji Photo Film Co., Japan) and software.

**6. Statistics:** Statistical analyses were not conducted. Mean ± standard deviation (SD) or individual data were reported. Data below the limit of quantification were reported as BLQ.

## II. RESULTS

## A. RADIOLABELED DOSE

**1. Radiochemical purity:** The radiochemical purities of the stock solution and Day 1 and 2 dose formulations as determined by TLC were 99.7%, 99.9%, and 99.7%, respectively.

**2. Dose levels:** The mean actual doses administered are presented in Table 2. The actual doses administered ranged between 99.6-102.0% of the nominal dose levels.

**B. CLINICAL SIGNS:** No treatment-related clinical signs of toxicity were observed.

## C. ABSORPTION/PHARMACOKINETICS

**1. Whole blood and plasma kinetics:** Whole blood and plasma kinetics are presented in Table 3. The maximum concentration of [$^{14}$C]-dicamba in whole blood was observed at 0.5 hours after the second dose (35.6 µg-equivalents/g; 161 nmol equivalents/g) and declined steadily to the final 6-hour sample (1.15 µg-equivalents/g; 5.20 nmol equivalents/g). Similarly, the maximum concentration of [$^{14}$C]-dicamba in plasma was observed at 0.5 hours after the second dose (53.4 µg-equivalents/g; 242 nmol equivalents/g) and declined steadily to the final 6-hour sample (1.81 µg-equivalents/g; 8.19 nmol equivalents/g). Plasma concentrations were consistently greater than those in whole blood.

| **TABLE 3.** Mean (± SD) concentrations of total radioactive residues in whole blood and plasma after two oral doses of [$^{14}$C]-dicamba at 75 mg/kg/day to male rats. [a] | | | | |
|---|---|---|---|---|
| **Time post-dose (hours)** | **Concentration** | | | |
| | **µg-equivalents/g** | | **nmol equivalents/g** | |
| | **Whole blood** | **Plasma** | **Whole blood** | **Plasma** |
| **0.5** | 35.6 ± 5.1 | 53.4 ± 7.8 | 161 ± 23 | 242 ± 35 |
| **1** | 28.6 ± 6.7 | 43.3 ± 10.2 | 129 ± 30 | 196 ± 46 |
| **2** | 15.2 ± 6.2 | 23.1 ± 9.7 | 68.8 ± 28.1 | 105 ± 44 |
| **4** | 4.24 ± 1.01 | 6.45 ± 1.54 | 19.2 ± 4.6 | 29.2 ± 7.0 |
| **6** | 1.15 ± 0.96 | 1.81 ± 1.38 | 5.20 ± 4.34 | 8.19 ± 6.24 |

a Data were obtained from Tables 1 and 2 on pages 26-27 of MRID 51129104; n = 4 samples/time point.

**2.** **Duodenum kinetics:** Total amounts and concentrations of radioactivity in the duodenum sections and mincing solutions and scrapings are presented in Table 4.

**a.** **Duodenum sections:** In the duodenum sections, mean total radioactivity and mean concentrations of radioactivity were greatest at 0.5 hours after the second dose and declined with time. Mean total radioactivity in section A declined from 6.07 µg-equivalents to 0.185 µg-equivalents, section B declined from 2.39 µg-equivalents to 0.158 µg-equivalents, and section C declined from 2.31 µg-equivalents to 0.118 µg-equivalents. Mean concentrations of radioactivity in the sections also were greatest at 0.5 hours after the second dose with concentrations that ranged from 20.9 µg-equivalents/g to 11.6 µg-equivalents/g (94.6 nmol equivalents/g to 52.5 nmol equivalents/g) in sections A, B, and C and declined with time. Concentrations were still measurable at 6 hours after the second dose with concentrations ranging from 0.536 µg-equivalents/g to 0.504 µg-equivalents/g (2.28 nmol equivalents/g to 2.43 nmol equivalents/g). Concentrations in duodenum sections were greatest in section A (immediately after the stomach) and declined from sections A to B and generally declined from sections B to C between 0.5-2 hours after the second dose. At 0.5 hours, mean concentrations in section A were 20.9 µg-equivalents/g (94.6 nmol equivalents/g) declining to 6.62 µg-equivalents/g (30 nmol equivalents/g) at 2 hours. Mean concentrations in section B at 0.5 hours were 13.3 µg-equivalents/g (60.2 nmol equivalents/g) declining to 5.33 µg-equivalents/g (24.1 nmol equivalents/g) at 2 hours. Mean concentrations in section C at 0.5 hours were 11.6 µg-equivalents/g (52.5 nmol equivalents/g) declining to 5.92 µg-equivalents/g (26.8 nmol equivalents/g) at 2 hours. At 4 and 6 hours, mean concentrations were generally similar across all sections.

**b.** **Mincing solutions and scrapings:** Mean total amounts of radioactivity in section A ranged from 0.045 µg-equivalents to 0.001 µg-equivalents, section B ranged from 0.016 µg-equivalents to 0.001 µg-equivalents, and in section C ranged from 0.013 µg-equivalents to <0.001 µg-equivalents. Mean concentrations of radioactivity were greatest at 0.5 hours, ranging from 0.025 µg-equivalents/g to 0.007 µg-equivalents/g (0.114 nmol equivalents/g to 0.032 nmol equivalents/g) and declined with time, approaching the limit of quantification at 6 hours.

**TABLE 4.** Mean (± SD) concentrations of total radioactive residues in duodenum sections and mincing solution and scrapings after two oral doses of [$^{14}$C]-dicamba at 75 mg/kg/day to male rats. [a]

| Time post-dose (hours) | Concentration | | | | | |
|---|---|---|---|---|---|---|
| | Duodenum sections | | | Mincing solution and scrapings | | |
| | Total radioactivity (µg-equiv.) | Concentrations (µg-equiv./g) | Concentrations (nmol equiv./g) | Total radioactivity (µg-equiv.) | Concentrations (µg-equiv./g) | Concentrations (nmol equiv./g) |
| **0.5** | | | | | | |
| Section A | 6.07 ± 2.81 | 20.9 ± 8.4 | 94.6 ± 38.0 | 0.045 ± 0.036 | 0.025 ± 0.019 | 0.114 ± 0.085 |
| Section B | 2.39 ± 0.71 | 13.3 ± 4.4 | 60.2 ± 20.1 | 0.016 ± 0.006 | 0.009 ± 0.003 | 0.038 ± 0.015 |
| Section C | 2.31 ± 0.93 | 11.6 ± 2.0 | 52.5 ± 9.0 | 0.013 ± 0.004 | 0.007 ± 0.003 | 0.032 ± 0.013 |
| **1** | | | | | | |
| Section A | 4.39 ± 1.98 | 14.5 ± 7.6 | 65.6 ± 34.2 | 0.020 ± 0.009 | 0.011 ± 0.005 | 0.049 ± 0.022 |
| Section B | 1.96 ± 0.63 | 9.69 ± 2.53 | 43.8 ± 11.4 | 0.009 ± 0.003 | 0.005 ± 0.002 | 0.024 ± 0.009 |
| Section C | 2.03 ± 0.65 | 8.71 ± 1.75 | 39.4 ± 7.9 | 0.007 ± 0.003 | 0.005 ± 0.001 | 0.022 ± 0.004 |
| **2** | | | | | | |
| Section A | 2.22 ± 1.14 | 6.62 ± 2.71 | 30.0 ± 12.3 | 0.007 ± 0.002 | 0.004 ± 0.001 | 0.017 ± 0.004 |
| Section B | 1.25 ± 0.60 | 5.33 ± 2.03 | 24.1 ± 9.2 | 0.004 ± 0.002 | 0.003 ± 0.001 | 0.013 ± 0.004 |
| Section C | 1.31 ± 0.59 | 5.92 ± 2.16 | 26.8 ± 9.8 | 0.004 ± 0.002 | 0.003 ± 0.001 | 0.012 ± 0.006 |
| **4** | | | | | | |
| Section A | 0.574 ± 0.180 | 1.82 ± 0.38 | 8.24 ± 1.72 | 0.002 ± 0.001 | 0.001 ± 0.001 | 0.006 ± 0.002 |
| Section B | 0.357 ± 0.152 | 1.53 ± 0.50 | 6.92 ± 2.28 | 0.002 ± 0.001 | 0.001 ± 0.001 | 0.006 ± 0.002 |
| Section C | 0.363 ± 0.112 | 1.81 ± 0.87 | 8.19 ± 3.95 | 0.003 ± 0.002 | 0.002 ± 0.001 | 0.007 ± 0.005 |
| **6** | | | | | | |
| Section A | 0.185 ± 0.095 | 0.504 ± 0.290 | 2.28 ± 1.31 | 0.001 ± <0.001 | 0.001 ± <0.001 | 0.005 |
| Section B | 0.158 ± 0.154 | 0.534 ± 0.396 | 2.42 ± 1.79 | 0.001 ± <0.001 | 0.001 ± <0.001 | 0.005 |
| Section C | 0.118 ± 0.099 | 0.536 ± 0.440 | 2.43 ± 1.99 | <0.001 ± <0.001 | <0.001 | <0.001 |

a Data were obtained from Tables 3-8 on pages 28-33 of MRID 51129104; n = 4 samples/time point.

3. **Liver kinetics:** Liver kinetics are presented in Table 5. The mean concentration of radioactivity was greatest at 0.5 hours after the second dose (17.4 µg-equivalents/g; 78.7 nmol equivalents/g) and declined with time to 6 hours (0.98 µg-equivalents/g; 4.45 nmol equivalents/g).

**TABLE 5.** Mean (± SD) concentrations of total radioactive residues in liver samples after two oral doses of [$^{14}$C]-dicamba at 75 mg/kg/day to male rats. [a]

| Time post-dose (hours) | µg-equivalents/g | nmol equivalents/g |
|---|---|---|
| **0.5** | 17.4 ± 2.7 | 78.7 ± 12.3 |
| **1** | 13.7 ± 3.3 | 62.0 ± 14.8 |
| **2** | 7.75 ± 2.49 | 35.1 ± 11.3 |
| **4** | 2.88 ± 0.86 | 13.0 ± 3.9 |
| **6** | 0.983 ± 0.554 | 4.45 ± 2.51 |

a Data were obtained from Tables 10 and 11 on pages 35-36 of MRID 51129104; n = 4 samples/time point.

4. **Urine:** Urine data are presented in Table 6. Radioactivity was detectable in the urine at 1 hour following the first dose (1020 µg-equivalents/g), with the greatest concentration at 4 hours (4680 µg-equivalents/g). Radioactivity concentrations fell with time to a low of 111 µg-equivalents/g at 24 hours after the first dose. Following the second dose, radioactivity concentrations began to rise, with the greatest concentration at 2 hours after the second dose (6040 µg-equivalents/g) falling with time to the final collection at 6 hours (5570 µg-equivalents/g).

| TABLE 6. Mean (± SD) concentrations of total radioactive residues in urine samples after two oral doses of [$^{14}$C]-dicamba at 75 mg/kg/day to male rats. [a] | |
|---|---|
| **Sample (day + hours)** | **µg-equivalents/g** |
| **Day 1, 1 hour** | 1020 ± 1170 |
| **Day 1, 2 hours** | 4060 ± 4710 |
| **Day 1, 4 hours** | 4680 ± 2300 |
| **Day 1, 6 hours** | 2580 ± 1010 |
| **Day 1 24 hours** | 111 ± 32 |
| **Day 2, 1 hour** | 597 ± 62 |
| **Day 2, 2 hours** | 6040 ± 4240 |
| **Day 2, 4 hours** | 4460 ± 3040 |
| **Day 2, 6 hours** | 5570 ± 8550 |

a Data were obtained from Table 9 on page 34 of MRID 51129104; n = 4 samples/time point.

## III. DISCUSSION and CONCLUSIONS

**A. <u>INVESTIGATORS' CONCLUSIONS</u>:** Dicamba is rapidly absorbed following oral administration to male rats, with maximum mean radioactivity concentrations observed in whole blood, plasma, liver, and duodenum occurring at 0.5 hours post dose (first sampling time).

Mean concentrations of [$^{14}$C]-dicamba between duodenum sections A, B and C showed that section A (the first 0-2 cm after the stomach) contained the greatest concentrations at earlier time points (0.5-2 hours). At later time points (4 and 6 hours), mean concentrations between duodenum sections were generally similar, indicating a more uniform distribution of radioactivity within the duodenum.

**B. <u>REVIEWER COMMENTS</u>:** No clinical signs of toxicity were reported.

The maximum concentration of [$^{14}$C]-dicamba in whole blood was observed at 0.5 hours after the second dose and declined steadily to the final 6-hour sample. Similarly, the maximum concentration of [$^{14}$C]-dicamba in plasma was observed at 0.5 hours after the second dose and declined steadily to the final 6-hour sample. Plasma concentrations were consistently greater than those in whole blood.

In the duodenum sections, mean total radioactivity and mean concentration of radioactivity were greatest at 0.5 hours after the second dose and declined with time. Concentrations were still measurable at 6 hours after the second dose. Concentrations in duodenum sections were greatest in section A (immediately after the stomach) and declined from sections A to B and generally declined from sections B to C between 0.5-2 hours after the second dose. At 4 and 6 hours, mean concentrations were generally similar across all sections. Mean total radioactivity and mean concentrations of radioactivity in the mincing solutions and scrapings were less than those found in the duodenum sections and followed the same time course, approaching the limit of quantitation at 6 hours. Mean concentrations of radioactivity in the liver sections were greatest at 0.5 hours after the second dose and declined with time to 6 hours.

Radioactivity was detectable in the urine at 1 hour following the first dose with the greatest concentration at 4 hours. Radioactivity fell to the lowest concentration at 24 hours after the first dose. Following the second dose, radioactivity concentrations began to rise, with the greatest concentration at 2 hours after the second dose and falling with time to the final collection at 6 hours.

The Reviewers agree with the Investigators' conclusions.

This study is classified as **acceptable / non-guideline**.

**C. STUDY DEFICIENCIES:** The following deficiency was noted:

- Formulation analyses were not reported.

# DATA EVALUATION RECORD

DICAMBA

Study Type: OCSPP Non-Guideline; Gene Mutation Assay in Transgenic Mice

EPA Contract No. EP-W-16-018
Task Assignment No. 34-3-001
(MRIDs 51129105, 51129106, 51129107, 51129108, and 51129109)

Prepared for
Health Effects Division
Office of Pesticides Program
U.S. Environmental Protection Agency
2777 South Crystal Drive
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Prepared by
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10560 Arrowhead Dr., Suite 500
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| | | | |
|---|---|---|---|
| Primary Reviewer: | | Signature: | |
| Michael E. Viana, Ph.D. | | Date: | 08/21/2020 |
| Secondary Reviewer: | | Signature: | |
| Sarah E. Saucier, Ph.D. | | Date: | 08/27/2020 |
| Quality Assurance: | | Signature: | |
| Scott D. Studenberg, Ph.D., DABT | | Date: | 08/28/2020 |
| Project Manager: | | Signature: | |
| Michael E. Viana, Ph.D. | | Date: | 08/31/2020 |

This Data Evaluation Record may have been altered by the Health Effects Division subsequent to signing by CDM/CSS-Dynamac Joint Venture personnel. Contractor's role did not include establishing Agency policy.

**EPA Reviewer:** Sarah Dobreniecki **Signature:** Sarah Dobreniecki
**Risk Assessment Branch VII, HED (7509P)** **Date:** 9/16/2020
**Template version 02/06**

# DATA EVALUATION RECORD

**STUDY TYPE:** Gene Mutation Assay in Transgenic Mice; OCSPP Non-Guideline; OECD 488.

**PC CODE:** 029801 **DP BARCODE:** D458715

**TXR #:** 0058082

**TEST MATERIAL (PURITY):** Dicamba (89.8% a.i.)

**SYNONYMS:** BAS 183 H; SAN837 technical; 3,6-dichloro-2-methoxybenzoic acid

**CITATION:** Ueda, M. (2020) Dicamba techn. (BAS 183 H; SAN837 techn.): transgenic mice (Muta™Mouse) gene mutation assay. BioSafety Research Center, Inc., Shizuoka, Japan. Laboratory Study ID: 886458, March 13, 2020. MRID 51129105. Unpublished.

Ueda, M. (2020) Dicamba techn. (BAS 183 H; SAN837 techn.): dose range-finding study for transgenic mice (Muta™Mouse) gene mutation assay. BioSafety Research Center, Inc., Shizuoka, Japan. Laboratory Study ID: 886460, March 10, 2020. MRID 51129106. Unpublished.

Blum, M. (2020) Validation of an analytical method for the analysis of BAS 183 H (dicamba techn.) in powdered diet, CRF-1 (Oriental Yeast) using HPLC-UV (Control procedure 97/0267_03). BASF SE, Experimental Toxicology and Ecology, Ludwigshafen, Germany. Laboratory Study ID: 887672, February 3, 2020. MRID 51129107. Unpublished.

Bangert, L. (2020) BAS 183 H (Dicamba techn.): homogeneity and concentration control analysis in powdered diet CRF-1 (Oriental Yeast). BASF SE, Experimental Toxicology and Ecology, Ludwigshafen, Germany. Laboratory Study ID: 887680, January 30, 2020. MRID 51129108. Unpublished.

Wagner, I. (2020) BAS 183 H (Dicamba techn.): stability analysis in powdered diet CRF-1 (Oriental Yeast). BASF SE, Experimental Toxicology and Ecology, Ludwigshafen, Germany. Laboratory Study ID: 887679, February 11, 2020. MRID 51129109. Unpublished.

**SPONSORS:** Syngenta, Ltd., Jealott's Hill International Research Centre, Bracknell, Berkshire, UK
BASF SE, Ludwigshafen, Germany

**EXECUTIVE SUMMARY:** In a non-guideline, gene mutation assay (MRID 51129105), groups of seven male Muta™Mouse ($CD_2$-LacZ80/HazfBR) mice/dose level were administered dicamba (89.8% a.i., batch # P.MG2726410) in the diet at dose levels of 0, 1200, 3000, or 7000 ppm (equivalent to 0, 176.4, 431.1, and 924.9 mg/kg/day) for 28 consecutive days. After a three-day period for mutations to become fixed in the genomic DNA, the mice were euthanized on Day 31. A positive control group of seven male mice were administered *N*-ethyl-*N*-nitrosourea (ENU) in 1/15 mol/L sodium phosphate buffer (pH 6.0) by i.p. injection (dose volume 10 mL/kg) at a dose level of 100 mg/kg/day; two doses were given approximately 24 hours apart on Days 3 and 4. After ten days, these mice were euthanized on Day 14. The duodenum was examined for genomic DNA mutations induced by test substance exposure.

There were no effects of treatment on clinical signs of toxicity, absolute or relative (to body) duodenum weights, or necropsy or microscopic findings. There were no changes in absolute or relative (to body) duodenum weights in the ENU-treated group.

In the 7000 ppm group, there were decreases in body weight on Days 15 (↓6%) and body weight gain during Days 1-31 (−0.1 g treated vs 0.7 g control). Additionally, at 7000 ppm, food consumption was decreased by 22% during Days 1-3. In the positive control group, there was a decrease in body weight during Days 1-14 (↓8%).

Administration of dicamba in the diet did not increase mutant frequency. All mean values fell within the 95% confidence range ($14.4 \times 10^{-6}$ to $86.9 \times 10^{-6}$) calculated from the historical control data. The positive control (ENU) produced a marked 10.6-fold increase in the mutant frequency.

This study is classified as **acceptable / non-guideline**.

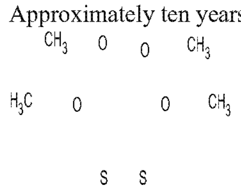
**COMPLIANCE:** Signed and dated Data Confidentiality, GLP Compliance, Flagging, and Quality Assurance statements were provided.

# I. MATERIALS AND METHODS

## A. MATERIALS

### 1. Test compound — Dicamba (BAS 183 H; SAN837 techn.)

**Description:** White solid
**Lot #:** P.MG2726410
**Purity:** 89.8% a.i.
**CAS # of TGAI:** 1918-00-9
**Expiration/storage:** Approximately ten years stored at room temperature
**Structure:**




### 2. Vehicle: Diet.

### 3. Test animals

**Species:** Mouse (male only)
**Strain:** $CD_2$-LacZ80/HazfBR (Muta™Mouse) transgenic mouse
**Age / weight at Day 1:** 11 weeks / 25.5-29.7 g
**Source:** Japan Laboratory Animals, Inc.
**Housing:** Individually in metallic mesh cages with an aluminum tray and bedding.
**Diet:** Powdered CRF-1 diet sterilized by irradiation (Oriental Yeast), *ad libitum*.
**Water:** Tap water, *ad libitum*.
**Environmental conditions**
- **Temperature:** 22.8-23.1°C
- **Humidity:** 47-57%
- **Air changes:** ≥12 / hour
- **Photoperiod:** 12 hours light/12 hours dark

**Acclimation period:** Approximately 8 weeks

## B. STUDY DESIGN

1. **Introduction:** It was stated that the aim of the present study was to investigate if dicamba could induce gene mutations (reporter gene: *lacZ*) in transgenic mice (Muta™Mouse) following dietary administration for 28 consecutive days by using the transgenic rodent (TGR) mutation assay. The TGR mutation assay was conducted according to the OECD TG-488 test guideline. The assay is used for the detection of *in vivo* mutations induced by the test substance by analyzing target organs. Genomic DNA is extracted from target organs of mice administered the test substance. The target gene is recovered from the genomic DNA by incorporation of the transgene into a λ bacteriophage or plasmid shuttle vector. Mutations are *in vivo* mutations that arise in the mouse and are detected by positive selection methods. The TGR mutation assay with *lacZ* bacteriophage mouse (Muta™Mouse) was considered appropriate for an evaluation of potential mutations because it has sufficient historical control data.

2. **In-life dates:** Start: October 29, 2019 End: November 28, 2019

3. **Animal assignment:** The mice were randomly assigned to the groups presented in Table 1 based on body weight on Day 1. It was stated that the weight range was not to exceed ±20% of the mean weight.

**TABLE 1:** Study design [a]

| Test group | Dietary concentration (ppm) | Compound intake (mg/kg/day) | # males [c] |
|---|---|---|---|
| **Control** | 0 | 0 | 7 |
| **Low** | 1200 | 176.4 | 7 |
| **Mid** | 3000 | 431.1 | 7 |
| **High** | 7000 | 924.9 | 7 |
| **Positive control** [b] | --- | 100 | 7 |

a Data were obtained from pages 34 and 43 of MRID 51129105.
b Positive control = 100 mg/kg/day *N*-ethyl-*N*-nitrosourea (ENU), administered by two i.p. injections approximately 24 hours apart.
c Seven mice/group were treated but only six mice/group were evaluated.
--- Not applicable

The first day of administration was designated as Day 1. The dietary formulations were administered during Days 1-28. The positive control mice were fed basal diet and were administered ENU by i.p. injection on Days 3 and 4 approximately 24 hours apart.

4. **Dose selection rationale:** The doses used in the present study were selected based on a dose range-finding study (MRID 51129406). A summary of this study is presented as Appendix 1 at the end of this DER.

5. **Diet preparation and analysis:** The dietary formulations were prepared by weighing an appropriate amount of the test substance (corrected for purity) and mixing it with basal diet using a mortar and pestle to form a premix. The premix was sieved (600 μM), transferred to a mixer, and combined with additional basal diet to yield the desired dietary concentration. The formulations were stored in sealed containers at room temperature and used within eight days.

A validation study (MRID 51129107) for an analytical method to detect dicamba in the basal diet is presented as Appendix 2 at the end of this DER.

Homogeneity and concentration analyses (MRID 51129108[1]) and stability analyses (MRID 51129109[2]) were presented in separate, concurrently-reviewed documents and are summarized here. Six samples (three samples from the top and bottom strata each) were randomly collected from each test substance formulation (one sample of the control/basal diet) and analyzed for homogeneity and concentration. Stability analyses were performed after completion of the definitive study (December 2019) on a 1000 ppm dietary

[1] Bangert, L. (2020) BAS 183 H (Dicamba techn.): homogeneity and concentration control analysis in powdered diet CRF-1 (Oriental Yeast). BASF SE, Experimental Toxicology and Ecology, Ludwigshafen, Germany. Laboratory Study ID: 887680, January 30, 2020. MRID 51129108. Unpublished.

[2] Wagner, I. (2020) BAS 183 H (Dicamba techn.): stability analysis in powdered diet CRF-1 (Oriental Yeast). BASF SE, Experimental Toxicology and Ecology, Ludwigshafen, Germany. Laboratory Study ID: 887679, February 11, 2020. MRID 51129109. Unpublished.

formulation; five samples were randomly collected following 0, 1, 3, 8, and 9 days storage at ambient temperature.

**Results**

**Homogeneity (%RSD):** 2.7-3.6%

**Stability (% of time 0):** 96.6% after 9 days storage at ambient temperature

**Concentration (% nominal):** 104.8-123.6%

The analytical data indicated that the mixing procedure was adequate and that the variance between nominal and actual dosage to the animals was acceptable. The variance between nominal and actual dosage was greater than normally accepted; however, because this is a special study and the high dose was intended to approximate a limit dose of 1000 mg/kg/day, the Reviewers do not consider this to be a major deficiency.

6. **Positive control preparation:** The positive control (ENU; *N*-ethyl-*N*-nitrosourea) was prepared by weighing the required amount and diluting it with 1/15 mol/L sodium phosphate buffer (pH 6.0) to yield a 10 mg/mL solution just prior to use. Formulation analyses were not reported.

7. **Statistics:** The following statistical analyses were performed. All statistical tests were two-tailed and significance was denoted at $p \leq 0.05$.

| Parameter | Analyses |
|---|---|
| Body weights, body weight gains, absolute and relative (to body) organ weights, and food consumption | Group means were analyzed with Bartlett's test for homogeneity of variance. If Bartlett's test was not significant ($p>0.05$), Dunnett's multiple comparison test was used to compare the treatment groups to the control. If Bartlett's test was significant ($p \leq 0.05$), Steel's test was performed to compare the treatment groups to the control. |
| Mutation frequency | Group means for the treatment groups and the control were analyzed as above. Group means for the control and positive control groups were analyzed with an F test for homogeneity of variance. If the F test was not significant ($p>0.05$), Student's t-test was performed. If the F test was significant ($p \leq 0.05$), an Aspen-Welch t-test was performed. |

The Reviewers consider the statistical analyses used appropriate.

## C. METHODS

1. **Observations:** The mice were observed for mortality and morbidity twice daily on weekdays during dosing and once daily on weekends and between dosing and euthanasia.

2. **Body weights and body weight gains:** The control and treated mice were weighed on Days 1, 3, 8, 15, 22, 29, and 31 (just prior to euthanasia). The positive control (ENU) mice were weighed on Days 1 and 14 (just prior to euthanasia).

3. **Food consumption and compound intake:** Food consumption (g/mouse/day) of the control and treated mice was determined by weighing the feeder on Day 1, 3, 8, 15, 22, and 29. It was stated that compound intake (mg/kg/day) was calculated but the method was not reported. (The Reviewers assume the calculations used the food consumption and body weight data and the nominal dietary concentrations.)

4. **Sacrifice and pathology:** The control and treated mice were euthanized by blood sampling/exsanguination under isoflurane anesthesia; the positive control mice were euthanized by exsanguination under isoflurane anesthesia. All mice were given a complete necropsy; the following tissues (X) were collected and the (XX) tissues were weighed.

| | | | | | |
|---|---|---|---|---|---|
| XX | Duodenum | X | Kidney | X | Lymph node (mesenteric) |
| X | Liver | X | Heart | X | Testis |
| X | Stomach | X | Bladder | X | Vas deferens/cauda epididymis |

Organs and tissues were processed as follows. Following processing, all tissues were stored in an ultralow temperature freezer.

a. **Duodenum:** The duodenum was examined macroscopically, weighed, and an approximately 6-cm section from the pylorus of the stomach was incised and the contents removed by flushing with isotonic saline. The duodenum was then cut into three segments approximately 2 cm long each. The section nearest the pylorus was fixed in 10% neutral-buffered formalin and the other two sections were frozen in liquid nitrogen. The fixed duodenum was processed routinely, stained with hematoxylin and eosin, and examined microscopically.

b. **Liver:** Two samples were obtained from the left lateral lobe and frozen separately in liquid nitrogen. The remaining lobes were placed in a storage bag, compressed, and frozen with a flat-bottomed metal container filled with liquid nitrogen. The remaining left lateral lobe was fixed in 10% neutral-buffered formalin.

c. **Stomach:** The stomach was incised and the contents removed by flushing with isotonic saline. A portion of the stomach (including forestomach and glandular stomach) was trimmed to approximately 4 × 10 mm and fixed in 10% neutral-buffered formalin. The remaining part was separated into forestomach and glandular stomach (two pieces), placed into separate storage bags, and frozen in liquid nitrogen.

d. **Kidney:** The capsule of the left kidney was removed, and two 1-2 mm thick pieces of the left kidney were sliced off and frozen separately in liquid nitrogen. The capsule of the right kidney was removed and the right kidney was fixed in 10% neutral-buffered formalin. Remaining parts were compressed in a storage bag and frozen with a flat-bottomed metal container filled with liquid nitrogen.

e. **Heart:** The heart was placed in a storage bag, compressed, and frozen with a flat-bottomed metal container filled with liquid nitrogen.

f. **Bladder:** The bladder was flushed with isotonic saline and frozen in liquid nitrogen.

g. **Lymph node:** Approximately one-third of the mesenteric nodes were fixed in 10% neutral-buffered formalin; the remaining nodes were placed in a storage bag, compressed, and frozen with a flat-bottomed metal container filled with liquid nitrogen.

h. **Testes:** The testes were frozen separately in liquid nitrogen.

i. **Vas deferens/cauda epididymis:** The tissue was incised and placed in a petri dish with 1.5 mL of cold Dulbecco's phosphate-buffered saline. The suspended germ cells were filtered with a 40-µM cell strainer and a 1-mL portion was frozen in liquid nitrogen.

5. **Extraction of duodenal genomic DNA:** Lysis buffer (3 mL; containing RNase) was added to a Dounce-type homogenizer and cooled with ice, then a frozen tissue sample was added and homogenized with a pestle. The homogenate was added to a chilled tube containing 0.5 M sucrose (3 mL) and centrifuged. The supernatant was removed and cooled RNase-containing Dounce buffer (3 mL) was added and mixed well to yield a nuclear/cell suspension. Next, a proteinase K solution (3 mL) was added to the suspension and gently mixed. This suspension was incubated at 50°C for approximately three hours until it became clear. An equal volume (6 mL) of phenol/chloroform (1:1; v:v) was added, mixed, and centrifuged. The upper (aqueous) layer was collected and transferred into another tube and another equal volume of phenol/chloroform was added, mixed, and centrifuged; this procedure was repeated a third time. The water layer was then extracted with an equal volume of chloroform/isoamyl alcohol (24:1; v/v). The water layer was transferred into another tube and genomic DNA was extracted by gradually adding ethanol. Extracted genomic DNA was transferred into a microtube containing 70% ethanol and incubated for 10 minutes. The mixture was centrifuged, the supernatant was removed, and the remaining ethanol was allowed to evaporate. TE buffer (50 µL; not described) was added and the tube was incubated overnight at room temperature to dissolve the DNA residues. The DNA-containing solution was stored refrigerated after preparation.

6. **Preparation of test strains, packaging genomic DNA, and plating:** *Escherichia coli C* (*lacZ–*, *gal E–*) was cultured for packaging. Packaging was performed by using a commercially-available kit. One tube of packaging extract (red tube) was thawed and 10 µL of genomic DNA solution (adjusted to a concentration of about 200-600 µg/mL) was added. The tube was mixed and incubated at 30°C for 90 minutes. Next, a tube of packaging extract (blue tube) was thawed and 10 µL was transferred to the red tube and mixed. The mixture was incubated at 30°C for 90 minutes, diluted with 700 µL of buffer, and mixed yielding the packaged DNA sample.

   The *E. coli* suspensions for calculating total number of plaques (for titer) and for calculating mutant frequency (for selection) were dispensed into tubes. Then, the entire volume of the packaged DNA sample was added to the tube for selection and mixed. The tube was incubated at room temperature for about 30 minutes to allow the phage to infect the *E. coli*. A small quantity (30 µL) of the infected suspension was diluted 10-fold with broth containing 10 mmol/L magnesium sulfate and 30 μL of the dilution was transferred to the tube for titering and stirred.

Magnesium sulfate solution (1M) was added to an agar solution at a volume ratio of 2:100 to make top agar for titration. A P-gal solution was added to the agar solution at a volume ratio of 2:100 to make the top agar for selection. Top agar was added to the tube for titering and mixed, and the contents were poured over a LB agar plate. Top agar was added to the tube for selection and the contents were poured over a LB agar plate in the same manner. The agar plates were incubated overnight at 37°C. In this single packaging, the number of plaques per animal were above 300,000; therefore, no further packaging was required.

7. **Plaque counting:** The number of plaques (N) in the plates for titration was counted and then the total number of plaques was calculated using the following equation:

$$\text{Total \# of plaques} = \mathrm{N} \times \left(\frac{300\mu\mathrm{L}}{30\ \mu\mathrm{L}}\right) \times \left(\frac{2700\ \mu\mathrm{L}}{30\ \mu\mathrm{L}}\right)$$

$$= \mathrm{N} \times 900$$

The number of plaques in the plates for selection was counted and recognized as mutant plaques.

The *lacZ* gene was selected as a reporter gene. The mutant frequency in a concerned organ was calculated by dividing the number of mutant plaques by the total number of plaques:

$$\text{Mutant frequency} = \frac{\text{\# of mutant plaques}}{\text{total \#of plaques}}$$

8. **Study validity:** The study was considered valid if the following conditions were met:

- The mutant frequency for the duodenum in the positive controls was markedly increased with a significant difference from the negative control; and
- The mutant frequency in the negative control should be within an acceptable range (mean ± 3 SD) relative to historical control data.

## II. RESULTS

A. **CLINICAL SIGNS:** No treatment-related clinical signs of toxicity were observed.

B. **BODY WEIGHTS AND BODY WEIGHT GAINS:** Body weight and body weight gain data are presented in Table 2. In the 7000 ppm group, there were decreases in body weights ($p \leq 0.05$; ↓6%) on Day 15 and body weight gain during Days 1-31 (−0.1 g treated vs. 0.7 g control). In the positive control group, there was a decrease in body weight during Days 1-14 (↓8%).

**TABLE 2.** Mean (± SD) body weights and body weight gains (g) in transgenic mice treated with dicamba in the diet for up to 28 days. [a]

| Day | Dose (ppm) | | | | |
|---|---|---|---|---|---|
| | **0** | **1200** | **3000** | **7000** | **ENU [b]** |
| 1 | 27.5 ± 1.4 | 27.4 ± 1.2 | 27.6 ± 1.2 | 27.4 ± 1.1 | 27.4 ± 1.0 |
| 3 | 27.2 ± 1.4 | 26.7 ± 1.3 | 27.0 ± 1.2 | 25.9 ± 1.2 | --- |
| 8 | 26.4 ± 1.7 | 26.4 ± 1.1 | 27.0 ± 1.2 | 25.9 ± 1.0 | --- |
| 14 [c] | --- | --- | --- | --- | 25.2 ± 0.4 |
| 15 | 27.8 ± 1.6 | 27.1 ± 1.1 | 27.2 ± 1.5 | 26.0 ± 0.9* (↓6) | --- |
| 22 | 27.3 ± 1.9 | 27.0 ± 0.8 | 27.2 ± 1.7 | 25.8 ± 1.2 | --- |
| 29 | 28.0 ± 2.0 | 27.4 ± 1.1 | 27.8 ± 1.9 | 26.5 ± 1.4 | --- |
| 31 | 28.2 ± 2.1 | 27.7 ± 1.3 | 28.7 ± 2.0 | 27.3 ± 1.6 | --- |
| BWG Days 1-14 | --- | --- | --- | --- | −2.2 ± 0.9 |
| BWG Days 1-31 | 0.7 ± 1.7 | 0.3 ± 0.9 | 1.1 ± 1.1 | −0.1 ± 1.0 | --- |

a Data were obtained from Appendix 1 on pages 49-50 of MRID 51129105. N = 7.
b Positive control = 100 mg/kg/day *N*-ethyl-*N*-nitrosourea (ENU), administered by two i.p. injections on Days 3 and 4.
c ENU mice euthanized on Day 14.
--- Not applicable
* Significantly different from control; p≤0.05.

**C. FOOD CONSUMPTION AND COMPOUND INTAKE:** Food consumption data are presented in Table 3. In the 7000 ppm group, food consumption was decreased (p≤0.05) by 22% during Days 1-3.

Compound intake data are presented in Table 1.

**TABLE 3.** Mean (± SD) food consumption (g/mouse/day) in transgenic mice treated with dicamba in the diet for up to 28 days. [a]

| Days | Dose (ppm) | | | |
|---|---|---|---|---|
| | **0** | **1200** | **3000** | **7000** |
| 1-3 | 3.7 ± 0.6 | 3.9 ± 0.5 | 3.8 ± 0.4 | 2.9 ± 0.5* (↓22) |
| 3-8 | 3.8 ± 0.6 | 3.9 ± 0.4 | 4.0 ± 0.5 | 3.2 ± 0.4 |
| 8-15 | 3.7 ± 0.4 | 3.5 ± 0.7 | 3.2 ± 0.7 | 3.1 ± 0.4 |
| 15-22 | 4.0 ± 0.3 | 4.2 ± 0.4 | 4.2 ± 0.6 | 4.0 ± 0.4 |
| 22-29 | 4.3 ± 0.5 | 4.3 ± 0.4 | 4.3 ± 0.6 | 4.0 ± 0.5 |

a Data were obtained from Appendix 3 on page 58 of MRID 51129105. N = 7.
* Significantly different from control; p≤0.05.

## D. SACRIFICE AND PATHOLOGY

1. **Duodenum weights:** There were no effects of treatment on absolute or relative (to body) duodenum weights in the dicamba- or ENU-treated groups.

2. **Gross pathology:** There were no treatment-related effects noted at necropsy. The only macroscopic finding was a single, 4-mm white nodule in the right lung of a control mouse.

3. **Microscopic pathology:** No microscopic findings were reported.

**E. MUTANT FREQUENCY:** Mutant frequency data are reported in Table 4. Administration of dicamba in the diet did not increase mutant frequency. All mean values fell within the 95% confidence range ($14.4 \times 10^{-6}$ to $86.9 \times 10^{-6}$) calculated from the historical control data.

The positive control (ENU) produced a marked (p≤0.05) 10.6-fold increase in the mutant frequency.

**TABLE 4.** Mean (± SD) mutation data in the duodenum of transgenic mice treated with dicamba in the diet for up to 28 days. [a]

| Parameter | Dose (ppm) | | | | |
|---|---|---|---|---|---|
| | **0** | **1200** | **3000** | **7000** | **ENU [b]** |
| Plaque forming units | 988,500 ± 286404.1 | 1,208,850 ± 365,388.7 | 1,054,050 ± 242,978.9 | 895,950 ±. 307,626.7 | 654,900 ± 159,210.7 |
| # of mutants | 75 ± 34.7 | 85 ± 48.0 | 81 ± 40.5 | 57 ± 12.6 | 520 ± 116.6 |
| Mutant frequency ($10^{-6}$) | 75.3 ± 18.3 | 66.4 ± 18.4 | 75.3 ± 28.7 | 65.3 ± 9.9 | 799.2 ± 57.8* |

a Data were obtained from Table 1 on page 47 of MRID 51129105. N = 6.
b Positive control = 100 mg/kg/day *N*-ethyl-*N*-nitrosourea (ENU), administered by two i.p. injections on Days 3 and 4.
* Significantly different from control; p≤0.05.

## III. DISCUSSION and CONCLUSIONS

**A. <u>INVESTIGATORS' CONCLUSIONS</u>:** The negative control value was within the acceptable range of the historical control data and thus considered as valid. The mutant frequencies in the duodenum of the animals treated with Dicamba techn. (BAS 183 H; SAN837 techn.) did not show any increases as compared to the concurrent negative control value. All individual as well as group values were also within the historical control data.

The mutant frequencies in the duodenum in the positive control group, which was treated with *N*-ethyl-*N*-nitrosourea (ENU), increased and these increases were statistically significant compared with those of the negative control group. Therefore, the present study was judged to be properly conducted.

Considering all information available, including statistical analysis, it was concluded that Dicamba techn. (BAS 183 H; SAN837 techn.) did not induce gene mutations in the duodenum of transgenic mice (negative) under the conditions in this study.

**B. <u>REVIEWER COMMENTS</u>:** The Reviewers agree with the Investigators' conclusions.

There were no effects of treatment on clinical signs of toxicity, absolute or relative (to body) duodenum weights, or necropsy or microscopic findings. There were no changes in absolute or relative (to body) duodenum weights in the ENU-treated group.

In the 7000 ppm group, there were decreases in body weight (p≤0.05; ↓6%) on Day 15 and body weight gain during Days 1-31 (−0.1 g treated vs 0.7 g control). In the positive control group, there was a decrease in body weight during Days 1-14 (↓8%). Additionally, at 7000 ppm, food consumption was decreased (p≤0.05) by 22% during Days 1-3.

Administration of dicamba in the diet did not increase mutant frequency. All mean values fell within the 95% confidence range ($14.4 \times 10^{-6}$ to $86.9 \times 10^{-6}$) calculated from the historical control data. The positive control (ENU) produced a marked (p≤0.05) 10.6-fold increase in the mutant frequency.

This study is classified as **acceptable / non-guideline**.

**C. STUDY DEFICIENCIES:** The following deficiency was noted:

- The variance between nominal and actual dietary doses was greater than normally accepted (±15%).

**Appendix 1.** Dose range-finding study

In a non-guideline, dose range-finding study (MRID 51129106), groups of three male CD2F1/Slc (wild type for Muta™Mouse) mice/dose level were administered dicamba (89.8% a.i., batch # P.MG2726410) in the diet at dose levels of 0, 1000, 3000, or 10,000 ppm (equivalent to 0, 171, 434, and 1443 mg/kg/day) for 14 consecutive days. On Day 15, the mice were euthanized, necropsied, and the duodenum and liver were excised and weighed. The duodenum was examined microscopically.

All mice survived to scheduled euthanasia. There were no effects of treatment on clinical signs, body weights or body weight gains, food consumption, or gross or microscopic pathology.

At 10,000 ppm, absolute and relative (to body) liver weights were increased ($p<0.05$) by 32% and 29%, respectively, and absolute and relative duodenum weights were increased by 26% ($p<0.05$) and 28% (NS), respectively. Although there were no corroborating macroscopic or microscopic findings, the high dose for the main gene mutation assay study (MRID 51129105) was set at 7000 ppm and was expected to approximate the limit dose (1000 mg/kg/day) specified in OECD 488.

**Appendix 2.** Validation study

In an analytical method validation study (MRID 51129107), a method for the detection of dicamba (89.8% a.i.; batch P.MG2726410) in powdered CRF-1 basal diet (Oriental Yeast) was presented. Dicamba was incorporated into basal diet at two target concentrations of 1000 and 7000 ppm by grinding the test compound with a mortar and pestle and mixing an appropriate weight of the test compound with an appropriate weight of basal diet. The criteria for validation of the method included specificity, linearity of the calibration curve, accuracy, precision (repeatability), limit of quantification (LOQ) and limit of detection (LOD), and carry-over.

HPLC with a UV detection system was used to quantitate dicamba in the basal diet. Six matrix-matched calibration solutions were prepared from a stock solution of dicamba in acetonitrile (2.5 mg/100 mL, 5.0 mg/100 mL, 10.0 mg/100 mL, 15.0 mg/100 mL, 20.0 mg/100 mL, and 25.0 mg/100 mL).

The lowest calibration solution, solvent blank (acetonitrile), and basal diet extract were analyzed with a single injection. The method was considered specific if there was no response or a response <30% of the lowest calibration solution that corresponded to the retention time of dicamba in the calibration solution. The chromatogram of the calibration solution showed one peak (retention time [RT] approximately 3.5 minutes); chromatograms of the solvent blank and basal diet extract had no corresponding peaks. Therefore, the method was considered specific for dicamba.

The calibration solutions were analyzed with a single injection and the response (peak area) was correlated with the nominal concentrations by using regression analysis (without a weighting factor). The slope and intercept of the calibration curve, coefficient of determination ($r^2$), and back-calculated accuracies of the calibration solutions were calculated. The acceptance criteria were $r^2$ <0.990 and back-calculated accuracies of 100% ± 15%. The calibration curve was linear (slope = 2.821656; intercept = 0.032106), $r^2$ was >0.990 ($r^2$ = 0.999961), and the back-calculated accuracies all fell within the target range (−0.7% to 0.8%). Therefore, the calibration curve was accepted.

Accuracy and precision were determined in a single run. Five samples were taken from each of the dietary formulations (1000 and 7000 ppm) and extracted. Samples of the dietary formulations (5 g for the 7000 ppm formulation, 10 g for the 1000 ppm formulation) were placed in a 50-mL tube and 20 mL of 0.5 M sulfuric acid were added and shaken for 20 minutes. This mixture was extracted three times with 25 mL of acetonitrile for 30 minutes, the extracts were combined, made up to a final volume of 100 mL with acetonitrile, and filtered (0.2 µm). The extracts were analyzed with a single injection. Accuracy was calculated as:

$$\text{Accuracy} = \frac{\text{analyzed concentration}}{\text{target concentration}} \times 100$$

The relative standard deviation (%RSD) was calculated from the individual accuracies as:

$$\%\text{RSD} = \frac{\text{standard deviation}}{\text{mean accuracy}} \times 100$$

The acceptance criteria were that the mean accuracy fell within 70-110% of the nominal concentration (ideally 80-100%) and the %RSD for the formulations were ≤20% for precision. The mean accuracies were 99.9% and 104.7% of nominal and the %RSD were 4.5% and 1.9%, satisfying the accuracy and precision criteria.

For MRID 51129105, the lowest dietary concentration was 1000 ppm; therefore, the LOQ was defined as the lowest concentration of a sample where the accuracy and precision data fell within the tolerances. The LOQ was determined from the accuracy and precision data for the 1000 ppm formulation. The LOD was determined with a dicamba concentration of 2.0 mg/100 mL (20% of the LOQ or 200 ppm before extraction). A peak corresponding to the appropriate RT was clearly visible; therefore, the LOD was determined to be ≤200 ppm (from a 10-g sample).

Carry-over analysis was performed by injecting the highest calibration solution followed by two injections of basal diet extract. The acceptance criteria were to have no peaks corresponding to dicamba in the chromatograms or a peak <30% of the lowest calibration solution that corresponded to the retention time of dicamba in the calibration solution. There were no peaks corresponding to dicamba following injections of the basal diet extract; therefore, there was no carry-over from the injection of the highest calibration solution.

Stability analyses were not performed and were not considered necessary as a complete analytical run was performed in less than 24 hours.

It was concluded that the analytical method met the requirements for specificity, linearity, accuracy, repeatability, LOQ, and LOD for quantification of dicamba in powdered CRF-1 diet.